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Tracing organic carbon cycling in agricultural soils of different catch crop management systems by ^{13}C pulse labelling

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II. Abstracts in Portuguese and English

Abstract (English/Inglês)

The loss of nutrients from agricultural soils leads to environmental problems and the infraction of environmental laws. Catch crops are a type of cover crop that are used in rotations as a way of avoiding soil nutrients from leaching. These crops may enhance other properties of the soil regarding quality and biological activity. The aim of this thesis is to explore these benefits by comparing fallow soil, to that of a single-species catch crop (Mustard) and the soils of two multi-species catch crops (Mix4, containing four species, and a commercial mix TerraLife, containing thirteen), on the same agricultural soil in Lower Saxony, Germany.

A ^{13}C pulse labelling experiment was conducted to trace C through the atmosphere-plant-soil microbiome interphase. Net Ecosystem Production (NEP) of the different catch crops was calculated by monitoring gas fluxes. Soil microbial phospholipid fatty acids (PLFAs) were extracted, as a means of quantifying the relative amount of the microbial community and identifying relative differences among the soil samples.

NEP was significantly higher in the TerraLife soil when compared to the fallow soil and the Mustard suggesting that the higher the plant diversity, the higher the C fixation. The TerraLife soil presented higher extracted PLFA values, being significantly higher than the fallow soil at 0-10 cm for total PLFAs and fungal PLFAs, suggesting that higher plant diversity induces higher microbial biomass. And the soil microbial community structure seemed to present two different trends, with a higher Fungi/Bacteria ratio in the TerraLife and Mix4 soils and similar values for the Mustard and fallow soils.

The results suggest that multi-species catch crops provide higher C fixation rates and promote soil microbiota when compared to single-species crops or fallow soil. Further research that focuses on functional traits between individual catch crops and their microbiome is still needed in order to contrast and better interpret the information currently available on the subject.

Key words: soil microbiota, ^{13}C, PLFA, NEP, agricultural sustainability.

Resumo (Portuguese/Português)

A perda de nutrientes nos solos agrícolas provoca problemas ambientais e o incumprimento de leis de protecção do ambiente. As culturas de captura de nutrientes (“catch crops”) são culturas utilizadas nas rotações de cultivos para evitar a perda de nutrientes por infiltração. Estas culturas podem aumentar a qualidade ou a actividade biológica no solo. O objectivo desta dissertação é explorar aspectos relacionados com o ciclo do carbono ao comparar o efeito num solo agrícola da Baixa-Saxónia (Alemanha) de semear uma só espécie (Mostarda), semear mais do que uma (Mix4, mistura experimental de 4 espécies, e TerraLife, mistura comercial de 13 espécies) ou deixa-lo nu.

Para tal, foi dado às plantas um pulso de ^{13}C para fazer o seguimento do C na interfase atmosfera-planta-solo. A Produção Líquida do Ecossistema (NEP) foi calculada para as diferentes culturas de captura de nutrientes com recurso à monitorização dos fluxos de gases. Os ácidos gordos de fosfolípidos (PLFAs) dos microrganismos do solo foram extraídos para estimar a quantidade e diversidade relativas de distintos componentes do microbiota.

A NEP foi significativamente maior na TerraLife do que no solo nu e no da Mostarda, o que sugere que a maior diversidade de plantas pode estar a aumentar a fixação de C. O solo da TerraLife apresentou valores maiores do que o resto para PLFAs, significativamente maiores para PLFA total e PLFA de fungos, pudendo o aumento da diversidade das plantas estar a aumentar a biomassa microbiana. Observaram-se duas tendências na estrutura da comunidade microbiana; a TerraLife e a Mix4 tiveram maiores valores na razão fungo/bactéria; e a Mostarda e o solo nu, valores menores.

As "catch crops" com maior diversidade de plantas estudadas fixaram mais C da atmosfera e deram lugar a maior diversidade e abundância de microrganismos no solo que a de uma só espécie ou o solo nu. Ainda é preciso esclarecer as interacções específicas das plantas usadas com o microbiota do solo para comprovar os resultados e a interpretação dos mesmos.

Palavras chave: microbiota do solo, ^{13}C, PLFA, NEP, sustentabilidade agrícola.

III. Extended Portuguese abstract

Nos ecossistemas terrestres os solos são um local de circulação dos nutrientes entre matéria orgânica e inorgânica. Nos ecossistemas terrestres naturais, os microrganismos do solo são os principais responsáveis por essas transformações fechando o ciclo de nutrientes. Pelo que têm um papel fundamental na fertilidade dos solos. Contudo, nos solos de agricultura convencional a função dos microrganismos tem vindo a ser substituída pela intervenção humana, com a adição de nutrientes em formas mais ou menos disponíveis às plantas. Esta intervenção provoca uma mudança na reciclagem dos nutrientes, passando dum sistema com alta capacidade de regulação interna nos solos a um sistema aberto que precisa da adição regular de nutrientes para poder cobrir as necessidades nutricionais das plantas. Esta adição precisa de ser regular devido a perda da capacidade de retenção de nutrientes e carbono (C) no solo que seria fornecida pelo complexo sistema microbiano do mesmo.

A microbiota do solo é complexa e diversa, mas o foco nesta dissertação é nas bactérias e os fungos. Muitos estabelecem relações simbióticas com as raízes das plantas no solo, especialmente relevantes em ecossistemas pobres em nutrientes, influindo no seu desenvolvimento. Mas também os microrganismos que não estabelecem relações directas com elas (positivas ou negativas) influem de várias maneiras (por exemplo, bactérias que podem fixar azoto ou que competem com as plantas pelos mesmos nutrientes). Dos microrganismos simbióticos convém destacar os fungos micorrízicos arbusculares (AMF) pois estabelecem associações com a maioria de culturas agrícolas.

Dado que nem todas as plantas têm as mesmas necessidades ou estabelecem as mesmas associações com microrganismos, têm sido utilizadas rotações de cultivos em que diferentes plantas são semeadas umas a seguir às outras, com o objetivo de manter os solos férteis ao longo do tempo sem necessidade de adição excessiva de nutrientes. Além dos nutrientes, esta gestão de uso do solo permite manter o nível de matéria orgânica do solo (SOM) permitindo a existência de cadeias tróficas mais complexas e conferindo maior resiliência ao mesmo.

Com a adição massiva de nutrientes aos solos, os microrganismos perdem a funcionalidade que anteriormente tinham pelo que muda a composição microbiana e os nutrientes e a SOM não são retidos e atingem outros sistemas, nomeadamente as águas subterrâneas. Isto desestabiliza os ecossistemas que recebem os nutrientes provocando situações de eutrofização. As consequências provocam a perda de ecossistemas naturais, podendo impactar no curto e médio prazo nas economias locais. Para evitar estes problemas ambientais e as suas consequências, existem leis nacionais e supranacionais, notavelmente para os nitratos na Europa, que definem níveis máximos de substancias fertilizantes presentes nas águas de acordo com as suas funcionalidades.

Uma potencial solução para evitar estas perdas é a recuperação de culturas intercalares para a captura de nutrientes, chamadas em inglês “catch crops”. Estas plantas cultivam-se em épocas em que as condições não são boas para o cultivo de plantas comerciais em lugar de deixar o solo nu. As plantas utilizam os nutrientes presentes no solo para desenvolver o seu ciclo de vida evitando a sua perda a camadas inferiores, e quando morrem no inverno, pela sua decomposição, voltam os nutrientes ao solo. Existem numerosos estudos que defendem que além da maior eficiência do uso de nutrientes as "catch crops" podem estimular a actividade biológica do solo e melhorar as suas propriedades físicas e químicas (retenção de água, estrutura, etc.).

Este trabalho está enquadrado dentro de um projecto cujo objectivo é estudar as “catch crops” como ferramentas para manter a qualidade do solo e aumentar a produtividade (“Catch-Cropping as an Agrarian Tool for Continuing Soil Health and Yield Increase” ou “CATCHY”) desenvolvida por seis instituições alemãs parceiras. O objectivo específico deste trabalho é perceber a eficiência de

diferentes “catch crops” na reciclagem de C e o efeito no microbioma do solo. As hipóteses colocadas foram:

I. As “catch crops” com várias espécies fixam mais C no solo do que as de uma só espécie

II. Maior diversidade nas “catch crops” tem maior impacto positivo na biomassa microbiana do solo

III. A comunidade microbiana do solo apresenta diferenças em função da “catch crop” ser composta por uma só ou por várias espécies vegetais.

A experiência foi desenvolvida num Cambisolo Estágnico agrícola em Asendorf, na Baixa Saxónia (Alemanha). Neste campo, a rotação de cultivos, consistiu de fava, trigo de inverno e milho de forragem, e foram incluídas várias “catch crops” em diferentes parcelas de 9 m x 9 m. As “catch crops” estudadas nesta dissertação foram a Mostarda (*Sinapis alba* L.) e duas misturas de plantas; a “Mix4” com quatro espécies, e a “TerraLife”, uma mistura comercial com 13 espécies vegetais (tabela 2.2.).

Foi realizada uma marcação com ^{13}C para observar o translocação de C na interfase atmosfera-planta-solo. Para tal foi criado um compartimento estanque em redor da parte aérea das plantas no qual foi libertado $^{13}CO_2$, e monitorizada a sua fixação pelas plantas. Foram tomadas amostras de solo, plantas e ar antes e após a marcação. As amostras de solo tomaram-se a duas profundidades diferentes. A respiração do solo foi determinada em simultâneo com a toma de amostras de ar. Foram, também, tomadas amostras de ar de diferentes profundidades do solo.

Das amostras de solo, além das análises químicas, foram extraídos ácidos gordos de fosfolípidos (PLFA) das membranas dos microrganismos do solo. Estas moléculas servem como indicadores da presença e abundância de microrganismos vivos no solo e algumas são utilizadas como bio-indicadores da presença de taxa específicos. Esta técnica tem ajudado no estudo da estrutura das comunidades microbianas sendo hoje em dia um método comum. A extracção dos PLFAs consiste em romper as membranas dos microrganismos e isolar os fosfolípidos do resto de lipídos extraídos num primeiro momento; e hidrolisar, metilar e separar os ácidos gordos dos grupos polares aos quais estão unidos, obtendo como resultado ésteres metilados de ácidos gordos (FAMEs). Finalmente estes são medidos mediante cromatografia de gases.

Através da marcação com $^{13}CO_2$, foi observada a presença de ^{13}C a diferentes profundidades no solo e pôde-se calcular o tempo de residência média (MRT) em cada uma delas para as diferentes culturas. Observou-se que o ^{13}C teve um maior MRT a 60 cm de profundidade nas “catch crops” com várias espécies, podendo ser devido a uma maior reciclagem da parte da microbiota do solo dos exsudados das plantas. Também o MRT do ^{13}C obtido dos dados de fluxo de CO_2 do solo para a “TerraLife”, é maior do que nas outras “catch crops” o que sugere que a maior diversidade de plantas pode estar a induzir uma maior capacidade do solo para reter o C recentemente fixado da atmosfera.

Também se pôde estudar a incorporação e presença de carbono nas plantas e a produção líquida do ecossistema (NEP) para cada uma das “catch crops”. A “TerraLife” apresentou a maior taxa de marcação e maior NEP o que sugere que a maior diversidade de espécies vegetais das “catch crops” poderá ter vantagens para a incroporação de carbono no ecossistema.

Mediante a extracção de PLFAs das amostras do solo das diferentes “catch crops”, foi possível compará-las em função da quantidade total de PLFAs extraídos. A “TerraLife” teve um valor maior do que o resto, mas só significativamente diferente em relação ao solo nu. Integrando todos os resultados, parece muito provável que a maior heterogeneidade de espécies vegetais possa estar a potenciar

diferentes fungos e bactérias em diversos nichos ecológicos do solo, justificando uma maior abundância de biomasssa microbiana.

Também se pôde analisar a fracção dos PLFAs correspondentes exclusivamente a bactérias, fungos e o cálculo da razão fungo/bactéria para os diferentes solos. O solo que teve um valor maior para PLFA de fungos em baixa profundidade foi o da "TerraLife". Uma das diferenças que pode ter influenciado no respeitante à Mostarda é o facto de conter na mistura espécies de plantas que formam micorrizas. Contudo, também a maior diversidade de plantas pode ter potenciado a presença de fungos, tanto saprófitos como micorrizas, devido à menor especificidade a espécie vegetal e à capacidade de formar redes. A razão fungo/bactéria apresentou duas tendências diferentes. Por um lado, a "TerraLife" e a "Mix4" com valores maiores e evolução semelhante ao longo do tempo, e por outro, a Mostarda e o solo nu. Os valores maiores para as misturas de plantas sugere sistemas no solo com maior tendência a fechar os ciclos dos nutrientes e a preservação do C.

Dos resultados obtidos, as "catch crops" com maior diversidade de plantas estudadas fixaram mais C da atmosfera e deram lugar a maior diversidade e abundância de microrganismos no solo que a de uma só espécie ou o solo nu. É preciso continuar no estudo destas culturas, para obter mais dados e com eles maior confiança nas conclusões. Contudo, a partir dos resultados e da bibliografia existente, as "catch crops" com misturas de espécies têm um grande potencial na agricultura na redução da perda dos nutrientes, na maior incorporação de C no solo, na promoção do seu microbiota e nos serviços que fornecem às plantas e, por tanto, nos serviços que fornecem às pessoas.

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IV. Figure and table list

V. Abbreviation, acronym and symbol list (if necessary)

AIC, Akaike´s information criterion

AMF, arbuscular mycorrhizal fungi

BF_3, boron trifluoride

C, carbon

^{13}C, stable carbon isotope 13

CATCHY, "Catch-Cropping as an Agrarian Tool for continuing Soil Health and Yield Increase", name of the broad research project where this thesis was developed.

$CaCO_3$, calcium carbonate

CH_4, methane

$CHCl_3$, chloroform

CO_2, carbon dioxide

$^{13}CO_2$, carbon dioxide with isotope 13 carbon

$\delta^{13}C$, variation in isotope 13 carbon content

DW, dry weight

EMF, Ectomycorrhizal fungi

FA, FAs, fatty acid, fatty acids

FAME, FAMEs, fatty acid methyl ester, fatty acid methyl esters

FEP, fluorinated ethylene propylene, material of which the plastic pipes for air samples is made of.

GC, gas chromatograph, gas chromatography

HOS, high organic standard

H_2SO_4, sulphuric acid

IRMS, infrared mass spectroscopy

K^+, potassium cation

LMM, linear mixed effects model

MeOH, methanol

Mix4, multispecies catch crop used in our field experiment, containing field mustard, Egyptian clover, sorghum and Phacelia (check table 2.2. for more details)

MRT, mean residence time

N, nitrogen

$Na_2{}^{13}CO_3$, sodium carbonate containing isotope 13 carbon

NaCl, sodium chloride

NaOH, sodium hydroxide

NEP, net ecosystem production

NH_4SO_4, ammonium sulphate

NLFA, NLFAs, neutral lipid fatty acid, neutral lipid fatty acids

NMDS (ordination method), non-metric multidimensional scaling

N_2O, nitrous oxide

NPP, net primary production

OC, organic carbon

OM, organic matter

P, phosphorus

PLFA, PLFAs, phospholipid fatty acid, phospholipid fatty acids

ppm, parts per million

qPCR, quantitative polymerase chain reaction (method in molecular biology related to DNA replication)

rpm, revolutions per minute

Rs, respiration

R_{VPDB}, standard $\delta^{13}C$ value 0 used to measure stable carbon isotope ratio.

S, sulphur

S2-, sulphur anion

SE, standard error, standard errors

SOC, soil organic carbon

SOM, soil organic matter

SPE, solid phase extraction

TerraLife, multispecies catch crop used in our field experiment containing 13 plant species. The complete commercial name is "TerraLife-MaisPro TR Greening©"

TN, total nitrogen

UAN, urea and ammonium nitrate solution

VPDB, Vienna Pee Dee Belemnite, fossil whose $\delta^{13}C$ content is used as $\delta^{13}C$ value 0 when looking at carbon stable isotope allocation.

Other abbreviations used in the text which are not included in this list, correspond to the International System of Units.

1. Introduction

1.1. Current situation

1.1.1 Soils

Soils are fundamental to life on Earth (FAO, 2015). They are the interface between the organic and inorganic world in terrestrial ecosystems and, as such, they provide important ecosystem services. Ecosystem services are the benefits ecosystems provide humans and they can be divided into provision services (e.g. water), regulating services (e.g. disease control), cultural services (e.g. sense of belonging) and supporting services (e.g. nutrient cycling) (Millennium Ecosystem Assessment, 2003). Soil biota is the key driver of many of these, it has been shown that soil biodiversity influences ecosystem functions and it is generally accepted that it confers soil resistance and resilience to environmental changes (Pascual *et al.* 2015). However, the role soil microorganisms play has long been disregarded in agricultural systems because their function was replaced by human inputs or actions, like the use of fertilisers and pesticides or soil tillage (Barrios, 2007). Thus, the intensification of agriculture has reduced the complexity of soil food webs and biodiversity (Pullman *et al.* 2012, Tsiafouli *et al.* 2015), making those soils fragile and dependant of high external input. Studying and understanding the potentialities of microorganisms´ diversity and function for internal regulation in soils, may offer some ideas for more sustainable ways of producing food as well as preserving human well-being in the medium to long term (Barrios, 2007).

Nutrient cycling, a supporting ecosystem service, has been altered in soils with agricultural intensification. Nitrogen (N), phosphorus (P) and carbon (C) biogeochemical cycles have been altered in the last century due to human activity (Galloway *et al.* 2008, Morris and Blackwood 2015). N fixation through the Haber-Bosch reaction increased the inputs of reactive plant available N, one of the leading causes to the fast intensification in agricultural practices (Gorman 2013). Similarly, the natural abundance of plant available P is low, requiring constant input of nutrients with available P, especially when there is no *in situ* regulation of these compounds. Intensive application of N and P fertilizers causes environmental problems by leaching of excess nutrients to aquatic systems (Smith *et al.* 1999, Pearl 2016). The combustion of fossil fuels and the higher livestock production has increased the global greenhouse gas concentrations of CH_4, CO_2 and N_2O in the atmosphere in 35% (Le Quéré *et al.* 2016). This acceleration of the biogeochemical cycles threatens the ecological equilibrium that allows the survival of the human species, among many others.

As an important interface in terrestrial ecosystems, the role of soils as source or sink for greenhouse gases is crucial. Soils contain the largest terrestrial organic C reservoir holding 2060± 215 Pg C in the upper two meters (Batjes 2016). The use of cover crops in agricultural soils is a sustainable practice that can preserve or enhance C sequestration in agroecosystems (Poeplau and Don 2015).

1.1.2. Soil microbiology and plant development

Soil biota is the major driver in terrestrial biogeochemical nutrient cycles. Microorganisms in the soil play an important role by seeking their own survival within a community under a set of given conditions (Morris and Blackwood 2015). Individual microorganisms have different physiologies and are able to adapt to certain environmental conditions and specific nutrient sources. The whole microbial community itself responds to perturbations or changes of their habitat, shifting its composition and supporting the better adapted species.

The term soil microbiota will be used in the text mainly for bacteria and fungi, although other microscopic organisms are present in the soils; namely consumers (e.g. protozoans, rotifers) and some types of unicellular algae (Coleman and Wall 2015). Soil microbiota are in close contact with plant roots and are important regulators for plant nutrition, especially in nutrient poor environments (van der Heijden, 2008). Symbiotic microorganisms can have positive direct effects on plant growth and crop yields (van der Heijden, 2006). Mycorrhizal fungi are a group of symbionts that establish relationships with a great diversity of terrestrial plants. They provide a higher supply of limiting nutrients and, often, resistance to biotic or abiotic stress, in exchange for photosynthetic carbohydrates from the plant (van der Heijden, 2008, Balestrini 2015). There are a great number of different mycorrhizal fungi in the plant-soil system which can be divided into two main groups based of the position of the fungal structures (in the root tissues or mainly on the outside) and the taxonomy of the plant partners. These main groups are endomycorrhizal fungi, of which about two-thirds are arbuscular mycorrhizal fungi (AMF), and ectomycorrhizal fungi (EMF) (Balestrini 2015).

AMF belong to the Glomeromycota division, with about 220 species, and they are obligate biotrophs (they need their host plant to develop). They are important in agricultural systems because they form associations with most of the economically relevant crops enhancing their productivity (Balestrini 2015). EMF belong mainly to the Basidiomycota division but there are some belonging to the Ascomycota division too, with at least 6000 species. Their mycorrhizal mycelium is connected to an extended hyphal network in the soil involved in the search of nutrients and water. They are present in many different biomes and they are known for their fruiting bodies, some of which are edible (e.g. truffles or boletes) (van der Heijden, 2008, Ballestrini 2015).

Besides the various positive direct impacts on plant health, some soil microorganisms can also produce negative direct effects to the plant-soil system. Fungi and bacterial diseases are still the major cause for annual crop losses in low diversity agricultural systems (van der Heijden, 2008).

Apart from the direct effect of plant nutrition, microorganisms in the soil provide a number of indirect effects to plants. Detritus feeding microorganisms degrade plant residues and thereby transfer organic compounds inaccessible to plant nutrition into mineral compounds which can be taken up by plants. Among these microorganisms, bacteria and fungi that break down complex insoluble organic matter polymers containing nutrients into dissolved and plant accessible nutrients can be found. By fixing nutrients within their biomass, soil organisms prevent leaching losses to aquatic systems acting as a kind of nutrient buffer. Also many non-mycorrhizal fungi exudate organic acids for external digestion and leave available some of the more simple organic matter produced. There are other microorganisms that supress plant diseases because they produce specific antibiotic metabolites that benefit themselves (van der Heijden, 2008). Microorganisms can also have negative effects for plants indirectly, like the reduction of soil nutrients in certain conditions (competition with microbes for nutrients) or the mobilisation of N from the soil (van der Heijden, 2008).

Because of all these different direct and indirect effects on plants, it is often difficult to determine or even estimate the effects microorganisms have on plant productivity (Wardle *et al.* 2004). The aim of a sustainably managed soil should be, therefore, the establishment of a highly diverse and efficient soil microbial community with positive impacts on the plants and the suppression of potential pathogens. In this direction, concepts like ecological intensification or soil ecological engineering express the idea that agricultural systems that serve human interest as well as reducing negative environmental impacts are needed (Bender *et al.* 2016).

1.1.3. Crop rotations and biodiversity

Crop rotations have been used since the beginning of agriculture and often, when including legumes in the rotation, as a way of increasing available N compounds in the soil (Smith *et al.* 2015). In industrialised agriculture, cropping systems have been often simplified with a dramatic loss of biodiversity in the agroecosystems. The benefits of crop rotation to soil fertility depend on management, climatic and soil conditions. Crop rotation's direct impact alone may be difficult to quantify. However, together with other management strategies such as catch cropping or green manure application, crop rotations may have a fundamental impact on long-term soil fertility (Coleman 1995) and it is widely accepted that they improve soil functioning (Buldock, 1992). Different crops can explore different soil niches for nutrients (e.g. via different rooting depth), have different nutrient demands and have different soil symbiotic partners and pests. So, by changing the plant species we sow, we may have positive impact on soil health in the long-term.

McDaniel *et al.* (2014) analysed a great number of studies to elucidate the effect of adding one or more crops in rotation to a monoculture and observed that there was a general increase in the soil C and N pools when plant diversity also increased. The authors concluded that the close association of roots, microorganisms and minerals could be affecting the saprophyte community and encouraging physical SOM stabilisation. Depending on the type of plant, there are different effects on the soil and its microbiome. Properties like the rooting depth, the quality and quantity of the plant litter added to the soil or the amount of root exudates (Smith *et al.* 2015) influence the soil microbial community directly. Plants can attract different organisms by altering the composition of root exudates released to the rooting zone (Raaijmakers *et al.* 2009). In this way, individual plant characteristics together with other species' and plant community characteristics influence the microbial community composition and its functions (Smith *et al.* 2015).

1.1.4. Soil organic matter and soil organic carbon

There is more C in soils than in the World's atmosphere and vegetation together and most of it is soil organic C (SOC) contained in SOM (FAO and ITPS, 2015). The SOC content serves as an approximate measure of the SOM, which contains about 50% of OC (FAO and ITPS, 2015). SOM controls a wide range of ecosystem services and responds rapidly to human-induced changes (FAO and ITPS, 2015). For instance, SOM influences soil structure and stability, the retention of water, promotes soil biodiversity and is a source of nutrients for plants (FAO and ITPS, 2015).

One of the major sources of organic carbon input in terrestrial ecosystems is through plant primary production. Through photosynthesis, plants fix CO_2 from the atmosphere to obtain the C to build their tissues. Then, by plant structure decomposition and root exudation, C is incorporated to the soil where soil fauna and microorganisms process the C rich molecules. SOC budget in the soil depends on various factors. Climate is one of these factors, because temperature and moisture limit or promote plant growth but also biomass mineralisation or decomposition due to soil microorganisms. The higher the temperature, given all other conditions are optimal, the faster the biological activity and thus the faster organic matter is mineralised. If the temperatures are lower, nutrient recycling is slower and promote SOC accumulation. The amount of water present in the soil limits (when high or low) or promotes (when high enough) the development of life, both for plants and microorganisms. So, as an extreme example, this is why in tropical rainforests there is a very high biomass production but, at the same time, a very high biomass mineralisation and, thus, low SOC. The complete opposite phenomenon happens in boreal and arctic ecosystems, where plant growth is slow and decomposition

is even slower (there is also low oxygen availability, that acts as a limiting factor), having as a result the highest SOC stocks on the planet.

Another factor is the quality of the litter of the above ground biomass, which will determine how easily it will be degraded in the soil. As well as regional climate, soil management is a factor that has a strong impact on quality and quantity of SOM, which normally decreases the higher the management intensity in conventional agricultural systems (Tsiafouli *et al.* 2015). The soil that will be used in the field study belongs to a conventional central European agricultural system, with relatively intense management and, thus, low quality and quantity of SOM.

All in all, the presence and even distribution of SOC in a given soil improves its chemical and physical properties, increasing nutrient retention and water holding capacity, making soils potentially more suitable for agricultural use (FAO and ITPS, 2015). So, not surprisingly, SOC and soil biodiversity have been directly related to the three aspects of food security; they could increase food availability, restore the productivity of degraded soils and make food production systems more resilient (FAO and ITPS, 2015). Given the current trend of soil degradation worldwide, maintaining and improving the SOC content of our soils is of extreme importance. 45% of all European soils are considered degraded when regarding indigenous OC content, and in agricultural soils, intensive land use is considered to have depleted their SOC down to 0-2%. (FAO and ITPS, 2015)

1.1.5. Political context and sustainability

Loss of C and N in agricultural soils, which has negative consequences for the environment, makes agricultural systems become less productive. High input agricultural systems have an excessively high leaching of N, P and K nutrients among others (Thorup-Kristensen *et al.* 2003), which leads to their accumulation in surface and ground waters. This boosts the excessive growth of opportunistic primary production species that cover water surfaces (such as algae), limiting the light and oxygen availability, which impede the normal development of the life forms that supported the original equilibrium of the newly polluted ecosystem. This process is known as eutrophication and has become a major threat to terrestrial aquatic ecosystems (Smith *et al.* 1999).

The low price of fertilisers and their intensive application for the short-term increase in crop production and profits are the main triggers for the intensive nutrient leaching. Apart from the environmental damage that it causes, there can also be negative socio-economic impacts and health related problems. As an example, socio-economic impacts are especially noticeable in areas that base their local economy on nature tourism. If their local environment is damaged, the local economy is damaged too. This situation has been raising concerns and has led to legal regulations in some areas of the world (Smith *et al.* 1999, Nett *et al.* 2011).

In 1991, the European Union approved the “Nitrates Directive” to protect waters of its member states through its transposition into each state’s legislation. The aim of the directive was to protect water quality across the EU, preventing nitrates from agricultural sources polluting ground and surface waters (European Union 2010). This directive is part of an EU environmental legislation framework. The transposition of the laws into national legislation establishes legal limits to N balance surpluses at the field scale (Nett *et al.* 2011). However, the enforcement of these measures is not easy (Liu *et al.* 2018).

In Germany, the EU has been increasing pressure on the federal government since 2012 because nitrate pollution in ground and surface waters has been increasing in recent years (Jobert 2016, Knight

2016, Schumacher 2016) and could lead to a fine for trespassing the established limits. Furthermore, a significant number of environmental organisations and companies in Germany (Bundesverbands der Energie- und Wasserwirtschaft e. V., 2018) directed a petition to the European Commission and the Federal Regional Government reporting this situation. They demand a real law enforcement of nitrate limits in continental waters, a tougher control on the fields and regarding the transport and distribution of animal slurry (used widely to fertilise fields) and, finally, a shift in subsidies to those food producers that run sustainable managed systems. In this petition they estimate the cost of removing nitrates from household water would increase the price of water for a domestic user in 50 to 60%. Removing nitrate from water would be very expensive for the public authorities who assume the cost (Jobert 2016), and it does not solve the costs of the environmental and potential socio-economic damage. This is why reduction of N pollution at the source is a better solution to solving the problem. Because of the complexity of soil processes and the numerous factors that intervene, there will not be one global miraculous solution; different locally developed management strategies must be found.

1.2. Management strategy

1.2.1. Catch cropping

A promising tool to improve soil nutrient cycling and reduce leaching losses are catch crops and green manures. These are crops used in temperate climates during seasons unsuitable for commercial crop growth (autumn and winter) instead of leaving the soil bare. Most of the following section is based on the review of Thorup-Kristensen *et al.* (2003).

Thorup-Kristensen *et al.* (2003) distinguish two types of cover crops: green manures and catch crops. Green manures refer to those cover crops that are planted with the objective of increasing soil fertility for following crops. The term catch crop, on the other hand, refers to a cover crop that is planted with the objective of preventing nutrient losses from the soil. By using these crops it is possible to prevent the loss of N to deeper soil layers, that would be inaccessible to plants, and thus avoiding excess leaching to aquifers (Thorup-Kristensen *et al.* 2003; Rinnofner *et al.* 2008; Dahlin and Stenberg 2010; Constantin *et al.* 2010; Nett *et al.* 2011; Piotrowska and Wilczewski 2012; Li *et al.* 2015).

Although these techniques have been used for a long time, and were a key tool in early intensive forms of agriculture (Thorup-Kristensen *et al.* 2003), these practices have been disappearing progressively due to the inexpensive access to fertilizers. However, recent environmental problems (as stated above) are increasing the relevance and importance of their use as an agrarian tool (Thorup-Kristensen *et al.* 2003).

There are a great number of factors that influence the productivity of the following crop. Some of these are soil type, temperature, sowing date, soil moisture, nutrients present in the soil at a given time, soil behaviour and characteristics of the commercial crop planted and other agricultural management practices (e.g. tillage vs. no tillage) are amongst the most important (Thorup-Kristensen *et al.* 2003; Net *et al.* 2011). This is why the effect of catch crop influence on the following commercial crop is difficult to establish. However, regarding N uptake exclusively, the main sources of this variation, according to Thorup-Kristensen *et al.* (2003), seem to be plant growth potential, N-uptake potential, root growth and the relationship between soil depletion and nitrate leaching loss. In any case, the N field effects of catch crops in the short term can be quite variable, which is why long-term data is necessary to test the potential benefit of catch cropping on the soil nutrient budget and the economic value of this management practice (Thorup-Kristensen *et al.* 2003; Constantin *et al.* 2010).

Some effects of catch crops have been proven at the field scale. Thorup-Kristensen *et al.* 2003, include a number of effects of catch crops apart from N retention:

1. Effects on nutrients other than N. Catch crops can increase P plant availability by converting from inorganic to available organic forms, especially by catch crop species that have long root hairs that acidify the rhizosphere or that are supported by mycorrhizal fungi association. The plant P is released slowly and is not as susceptible to adsorption and precipitation as inorganic P forms added in commercial chemical fertilisers. They can also affect potassium (K^+) content and that of other cations in the soil water by their active uptake or indirectly by taking up nitrate and sulphate anions that would bind to them. Sulphur (S) can easily be lost by leaching and has a similar behaviour to nitrogen in soils. Some catch crop species have a high uptake of S, like crucifers, which could influence its availability in the subsequent crops.
2. Effects on soil microbiological and faunal activity are evident in both the short and long-term. The effect generally depends on crop biomass inputs; the higher the inputs via root exudation, root turnover or leaf litter loss, the higher the increase in microbial activity.
3. Improvement of soil structure and water retention by catch crops, among other physical properties, may also improve commercial crop establishment and root development, and decrease soil losses. Regarding the soil's water content, when a catch crop is growing, it will take water from the field. This is why it is important to bear in mind the growing conditions and the growth period of these.

The possibility of propagating certain soil-borne pathogens is a common concern amongst farmers. Although there could be a potential risk, catch crops can have a suppressive effect instead if used as break crops via the release of certain organic compounds with toxic effects for certain pathogens or the stimulation of microbial activity increasing competition or antagonistic suppression of pathogenic organisms in the soil (Thorup-Kristensen *et al.* 2003).

As mentioned above, different catch crop species determine different soil effects. Catch crops can be sowed in the fields on their own (single-species) or together with other species (multi-species). The latter appear to be promising because they can combine the positive effects on the soil of various single species within a plant community. Leguminous green manures, apart from fixing atmospheric N, have a high N uptake, and growing them with non-legumes in mixtures may be a viable solution for improving, not only the N leaching loss potential, but also to reduce the amount of N fertilizer purchase and application in the fields. Crucifer catch crops have been seen to have deeper rooting systems than monocots, thus increasing the N uptake of the plant and the access to other nutrients that are lower in the soil profile. (Thorup-Kristensen *et al.* 2003). So, for example, a diverse catch crop mixture could include:

- Legumes, that can fix nitrogen via rhizobia (if required) and have a high N uptake potential;
- Crucifers, that have deep roots thus increasing nutrient acquisition, and
- Cereals which establish symbiotic relationships with mycorrhizal fungi and are used as green manures and nutrient storage.

Rooting density and rooting depth is increased in a mixture, so different nutrient niches could also be explored more intensively and mycorrhizal fungi would help extend further the exploration.

1.2.2. General project objectives and aim of the thesis

This thesis is part of the project "Catch-Cropping as an Agrarian Tool for Continuing Soil Health and Yield Increase" ("CATCHY") which is a consortium of six interdisciplinary project partners. The project aims to study the long-term effects of catch cropping on the stabilisation and improvement of soils, regarding biological, chemical and physical properties. The main objective of the project is "to employ catch cropping for developing innovative farming systems to preserve and improve soil fertility" (BONARES, 2015). With the project it is expected to achieve a better understanding of cause-effect relationships affecting soil fertility parameters, biological functions and interactions in soil and rhizosphere. It has a functional orientation, for it interacts with agronomic and economic factors, and the results are expected to provide information on system-optimised commercial catch crop mixtures and information for the development of guidelines for sustainable and efficient agronomic practices. The project is funded by the German Federal Ministry of Education and Research, it started on the 1st of April 2015 and it is intended to run in 3 blocks of 3 years each.

The specific aim of this thesis, is to understand the efficiency of C cycling in differently managed catch crop systems. Therefore, the study compares and evaluates the effect of single and multiple catch crop treatments, in comparison to fallow soil, all of which included in the same crop rotation. The focus is on understanding the carbon cycling and translocation through the atmosphere-plant-soil microbiome interphase. For this, we conducted an *in situ* ^{13}C pulse labelling experiment. We combined solid-state ^{13}C analyses in plants and soil, with CO_2 from soil respiration and phospholipid fatty acid (PLFA) analyses of the microbiome to compare its biomass and diversity. Regarding this, the following hypotheses have been raised:

I. Multi-species catch crops will fix more atmospheric C than single-specie catch crops.
II. High species diversity in the catch crops will have a positive impact on soil microbial biomass.
III. The soil microbiome composition will be different in multi-species catch crops when compared to single-specie catch crops.

2. Methods

2.1. Experimental site

The experiment was conducted at a long term experimental field in Asendorf, Lower Saxony, Germany (52º 45' 48.5" N, 9º 01' 27.8" E, WGS 84 reference system), which is maintained by the DSV breading station (Deutsche Saatveredlung AG - Lippstadt Bremen) in Asendorf. The soil can be classified as a Stagnic Cambisol, which is a moderately developed soil with stagnic properties and reducing conditions in some parts for some time during the year (IUGS, 2014). Cambisols provide excellent sites for agriculture, especially those with a high base saturation in the temperate zone which are among the most productive soils on Earth. The soil profile is presented in figure 2.1.

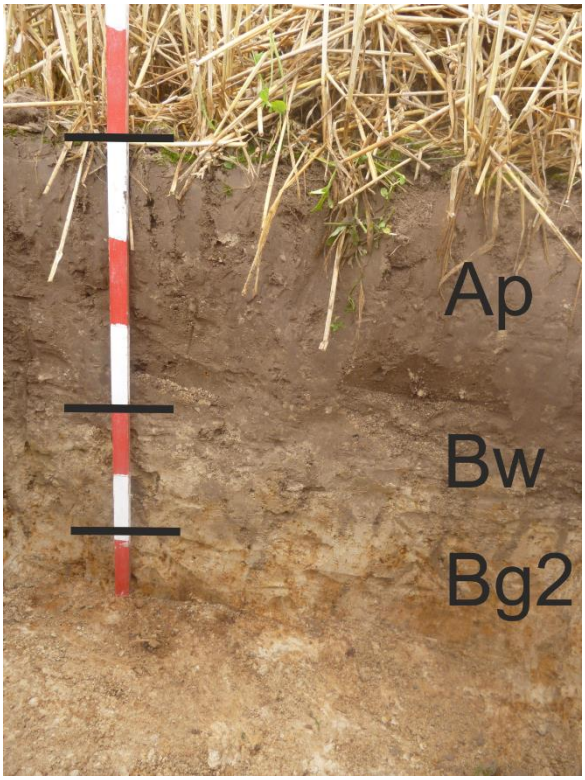

Figure 2.1. Asendorf soil profile. Photo taken by Norman Gentsch, Ap refers to the uppermost ploughed A horizon, Bw indicates the weathered B horizon and Bg2, the B horizon of different origin which presents stagnic conditions.

The location presents an oceanic temperate climate with a clear summer-winter temperature difference and precipitation spread out throughout the year. Climograph is shown in figure 2.2, where average temperature and precipitation per month of the last 34 years is presented.

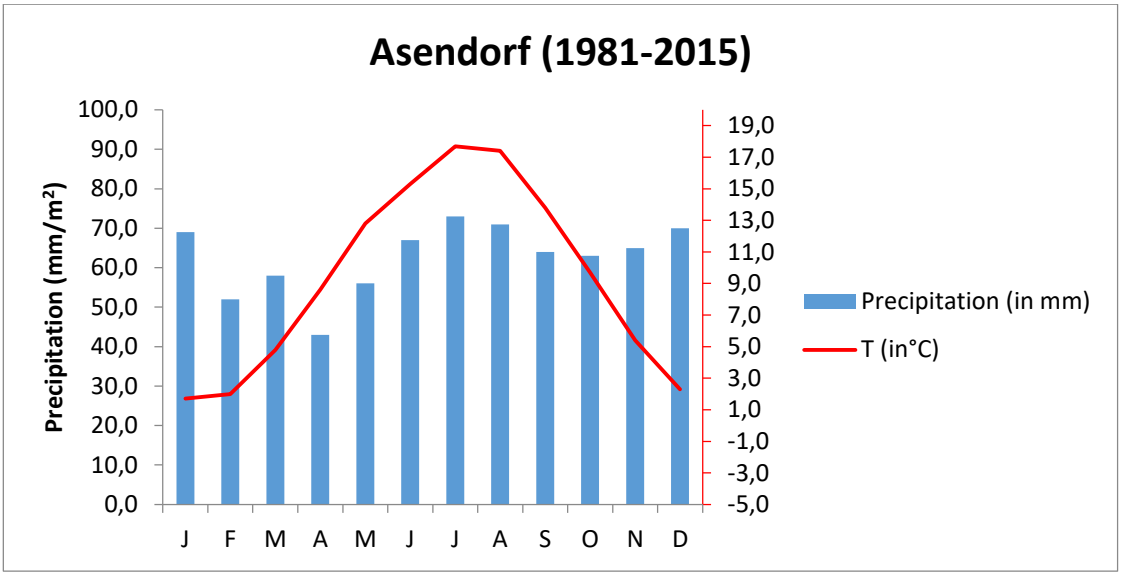

Figure 2.2. Month average temperature and precipitation in Asendorf from 1981 – 2015. Data from Hof Steimke station (Asendorf) and BASSUM Station.

Temperature during the sampling days is presented in figure 2.3. Air and soil temperature decreased during the second half of our sampling days.

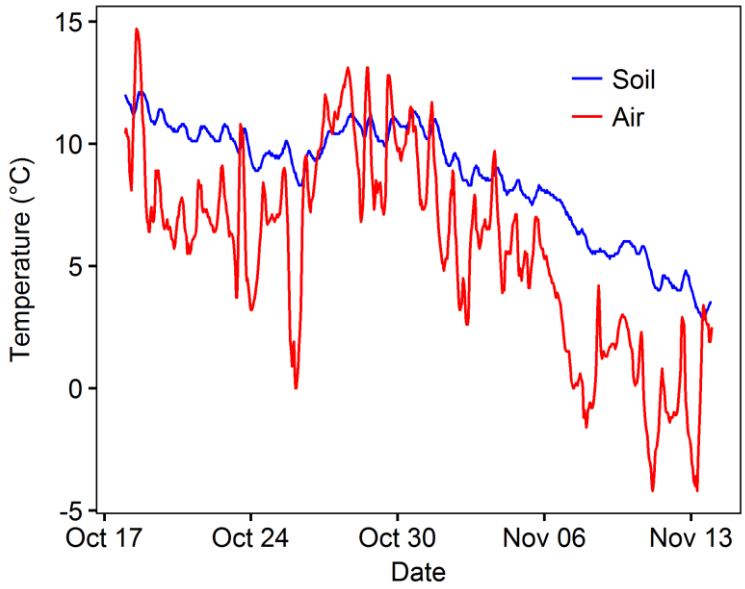

Figure 2.3. Air and soil temperatures in the field site during sampling period. Data from Asendorf field station (DSV). Soil temperature is for 15 cm depth.

2.2. Field experiment design

A fully randomised split-plot field experiment was conducted, where each plot had a size of 9 m x 9 m. The crop rotation in the field comprises field bean, winter wheat and silage maize, in that order. The catch crops were sowed after harvest of silage maize. Information on sowing, fertiliser and pesticide application before catch cropping is included in table 2.1.

Table 2.1. Dated field activities before catch cropping.

Date	Activity on the field site
March 2016	Winter wheat harvest
April 2016	Soil is mixed. UAN (urea and ammonium nitrate solution) is applied. 406 l/ha
May 2016	Silage maize is sowed. Fertilization with underfoot fertilizer "NP 18/46" Herbicides "Callisto" (100 g/l mesotrione) and "Gardo Gold" (S-metolachlor 312,5 g/l, terbuthylazine 187,5 g/l) are sprayed on silage maize plots to control dicotyledonous weeds in each case at a rate of 1.1 l/ha.
17th August 2016	Harvest of Silage maize
22nd August 2016	Sowing of catch crops
7th September 2016	UAN (urea and ammonium nitrate solution) fertilised application. 50kg/ha.

The catch crops of this study were mustard (*Sinapis alba* L.), an experimental mix ("Mix4") containing four different species and a commercial cover crop mix called "TerraLife-MaisPro TR Greening©" ("TerraLife") containing a mix of thirteen different species. The Mix4 species were selected in order to observe combined effect of different soil resource acquisition strategies. It includes a type of cereal, which establishes relationships with mycorrhizal fungi, a leguminous specie, that has a high N uptake capacity, a cruciferous specie, that has deep rooting, and a tough flowering plant often used in Germany as a cover crop (see section 1.2.1.). The idea of using a mix with fewer but representative specie types (regarding soil resource nutrient acquisition strategies) and a mix with higher diversity, is to see if there are significant differences amongst them, as well as comparing them with a single-specie catch crop and fallow soil. Description of the catch crop species composition, proportion of seed in each mix and sowing seed density are included in table 2.2.

Table 2.2. Experiment catch crops' species composition.

Catch crop mix	Species' common name	Proportion of seeds	Sowing seed density	Species' scientific name	Plant family
Mustard	Field mustard	100%	18 kg/ha	*Sinapis alba* L.	*Cruciferae*
Mix4	Sorghum	-	9 kg/ha	*Sorghum sudanense* (Piper) Stapf.	*Poaceae*
	Egyptian clover	-	7 kg/ha	*Trifolium alexandrinum* L.	*Fabaceae*
	Field mustard	-	4 kg/ha	*Sinapis alba* L.	*Cruciferae*
	Phacelia	-	5 kg/ha	*Phacelia tanacetifolia* Benth.	*Boraginaceae*

TerraLife-MaisPro TR Greening	Pea	38%		*Pisum sativum* L.	*Fabaceae*
	Sorghum	14%		*Sorghum sudanense* (Piper) Stapf.	*Poaceae*
	Phacelia	7%		*Phacelia tanacetifolia* Benth.	*Boraginaceae*
	Common flax	6%		*Linum usitatissimum* L.	*Linaceae*
	Crimson clover	6%		*Trifolium incarnatum* L.	*Fabaceae*
	Hungarian vetch	6%	35 kg/ha (of mix)	*Vicia pannonica* Crantz	*Fabaceae*
	Alsike clover	5%		*Trifolium hybridum* L.	*Fabaceae*
	Radish	5%		*Raphanus sativus* L.	*Cruciferae*
	Persian clover	4%		*Trifolium resupinatum* L.	*Fabaceae*
	Ramtil	4%		*Guizotia abyssinica* (L.f.) Cass.	*Asteraceae*
	Sunflower	2%		*Helianthus annuus* L.	*Asteraceae*
	Camelina	2%		*Camelina sativa* (L.) Crantz	*Brassicaceae*
	Safflower	1%		*Carthamus tinctorius* L.	*Asteraceae*

2.3. ^{13}C labelling

Pulse labelling of plants in artificial $^{13}CO_2$ atmosphere with subsequent tracing of ^{13}C in the plant-soil system is a common approach in ecosystem studies (Kuzyakov and Gavrichkova, 2010). There are different ways of adding the label into the plant-soil-microorganism system; one pulse labelling, continuous steady state labelling and series of repeated pulse labelling periods (Yao, 2015). We conducted a one-pulse labelling experiment, creating a $^{13}CO_2$ rich atmosphere where plants could take up the label via photosynthesis. By adding ^{13}C, the intention is to track the C from uptake through the plant system into the soil. By analysing the excess ^{13}C in plants, in soil air at different depths and in CO_2 "respired" from the soil, it is possible to infer the newly translocated C's utilisation. Three separate plots with the same catch crop were used as replicates. In each of these, an area of 75 cm x 120 cm was selected for a one-time pulse labelling with $^{13}CO_2$ (see area "a" figure 2.5). The same area was selected in three fallow soil plots (control) and were not labelled. The selected subarea in the field was labelled with a polyethylene sealed metallic frame of 75 cm x 120 cm x 110 cm (see figure 2.4). The real air volume inside the frame, after deducting the area occupied by the inside structure parts, was 997 200 cm³.

After installing the frame, the label was applied by adding 20 ml of 4M sulphuric acid (H_2SO_4) to 2 g $Na_2{}^{13}CO_3$ (based on Hafner *et al.*, 2012). The air inside the frame was kept circulating with a set of 4 cm x 4 cm x 2 cm vents (NB-BlackSilentPRO UntraSilent Premium Fan) connected to a 12 V rechargeable lead acid battery. The air flow each vent could produce was 188 330 cm^3/min. CO_2 concentration inside the frame was monitored with the LI-COR analyser (LI-8100 Automated Soil

CO_2 Flux System using LI-8100 20 cm Survey Chamber, LI-COR Inc., Lincoln, NE), connected to the frame through 3 mm flexible plastic pipes. The air was taken from the frame into the infrared gas analyser at a height of about 82 cm from the ground (28 cm from the top) and then released back in, forming a closed loop. To minimise differences on photosynthetic activity due to environmental conditions on the different labelling days, we provided artificial white light on the frame during the labelling with 6 fluorescent bulbs (*Philips* 36W/840).

The labelling was conducted on the 18th, 19th and the 24th of October 2016, when the catch crops were fully developed. On each day, we labelled three different catch crop plots.

Figure 2.4. Labelling polyethylene sealed metallic frame. LI-COR analyser. On top, lighting structure, LI-COR analyser (left, yellow case) and Survey Chamber (right).

2.4. Sampling

Samples of soil, plants and air were taken before labelling (day 0), on the days 1, 6, 11, 16 and 21 after labelling. On each sampling day, plant and soil samples were taken from 30 cm x 38 cm subareas (see subareas "b" in figure 2.5). Plant samples were taken by harvesting the total biomass in the subareas, including roots and shoots. Soil samples were taken at 0 – 10 cm and 20 – 30 cm depth. Additionally, soil respiration on each plot was measured using the LI-COR 20 cm Survey Chamber (LI-COR Inc., Lincoln, NE) attached to soil rings which were fixed to the ground (6 cm offset). The soil rings were installed 2 weeks before starting the measurements and plant material within them was removed and discarded (see subarea "c" in figure 2.5).

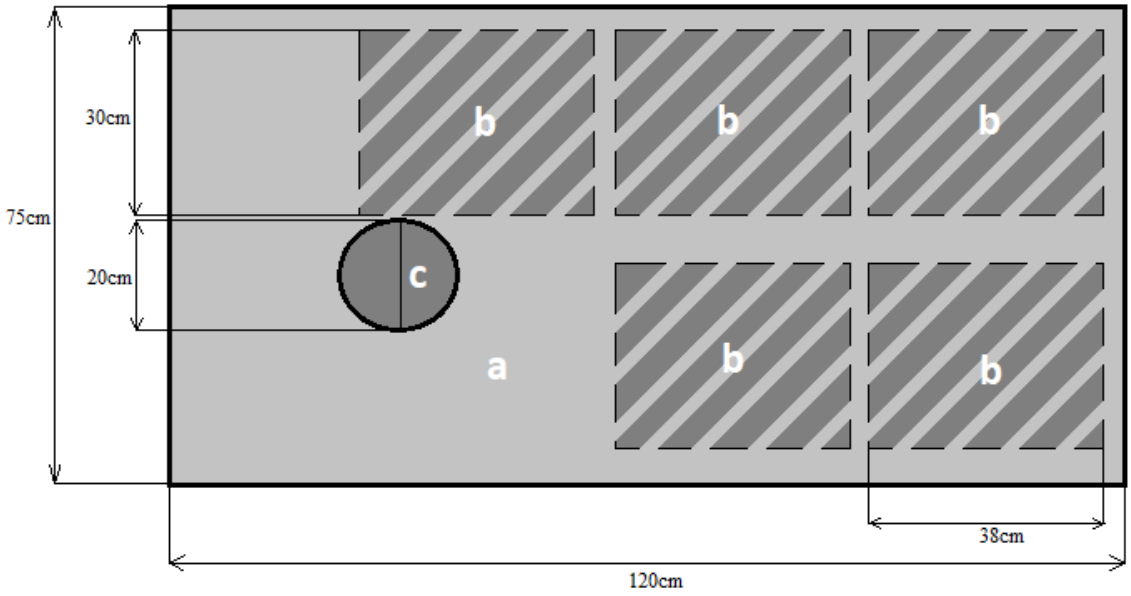

Figure 2.5. Schematic labelled area view from above. Area "a" corresponds to the total area within the labelling chamber; "b", the area where plant and soil samples were removed on each sampling day; and "c", the area where ring was installed for soil respiration measurements and gas sampling.

2.5. Soil respiration data

Soil respiration on each plot was measured using the LI-COR 20 cm Survey Chamber (LI-COR Inc., Lincoln, NE) attached to soil rings, which were fixed to the ground with a 6 cm offset, leaving a total volume inside the measurement chamber of 6 749.7 cm^3. The CO_2 efflux was monitored via the App (LI-8100A version 1.0.6) on an external mobile device connected to the LI-COR via Wi-Fi.

The soil respiration for a given treatment at day 0 was calculated from the fallow plot plus the factor by which the catch crops increase the respiration. The photosynthetic C uptake on the labelling days was calculated by the negative CO_2 flux inside the chamber plus the flux of the soil respiration. This C uptake is called Net Ecosystem Production (NEP) and it can be defined as the net organic carbon production via plant photosynthetic activity minus the C lost due to plant respiration and due to the degradation of its structures as a result of the activity of heterotrophic organisms in the soil (Kirschbaum *et al.*, 2001).

2.6. Gas samples

Three types of gas samples were taken. Those taken during ^{13}C label application, the ones taken during soil respiration measurements, and finally air from different soil depths. All samples were stored in pre-evacuated exetainers for isotope coupled gas chromatography analyses. The exetainers were evacuated, flushed with helium, and evacuated again for three times. During the labelling, samples were taken at four different time points: before the label was released, after the label was released and at two other time points after labelling.

During the soil respiration measurements, a t-connector with a septum was installed between the chamber and the instrument. 12 ml air samples were taken with an airtight syringe at increasing CO_2 concentrations (25, 50 or 100 ppm intervals).

Gas samples were taken at different soil depths (10, 30, 60 ± 2.5 cm). Soil air suction cups (ecoTech GmbH, Bonn, Germany) 2.5 cm in diameter were installed 2 weeks before start of the experiment. In order to minimise disturbances on the vertical soil column, the suction cups were installed by coring 2.5 cm wide holes at an angle of 45° until the appropriate soil depth. The suction cups were equipped with FEP pipes which were closed at the surface by two-way valves. Sampling occurred at the same time as the flux measurement by connecting a syringe to the valve. The first approximately 12 ml were discarded in order to get rid of the already accumulated gasses inside the pipes. The samples were injected in 12 ml pre-evacuated exetainers to be taken to the laboratory. Samples were then analysed with a gas chromatograph coupled to an isotope mass spectrometer (GC-Box coupled to a Delta plus XP system, Thermo Fisher Sientific, Bremen, Germany) to identify the CO_2 ppm and its isotopic composition.

2.7. Plant material

All plants within each subarea (represented as "b" in figure 2.5) were carefully harvested in order to avoid breaking the roots and we removed roughly the soil attached to them. In the lab, roots were washed with tap water and separated into roots and shoots. The plant material was coarsely cut by a scissor, dried for the first few days at ambient temperature and then in oven at 40 ºC. Once they were dry, they were weighed to calculate the dry weight per square metre, taking into account the sampling area. After this they were chopped finely with a blender and then ground using a ceramic ball mill (MM400, Retsch GmbH, Haan, Germany) at frequency 30/s for 1.5 minutes. From this, fine dust like particles were obtained for each sample, which were dried once more at 40 ºC overnight to remove any excess water. For element analyses the samples were weighed (scales used, Mettler-Toledo GmbH, Gießen, Germany) into tin boats (10-15 mg for roots and 5-10 mg for shoots). The C and N content as well as the $\delta^{13}C$ isotopic ratio was measured on an Elementar IsoPrime 100 IRMS (IsoPrime Ltd, Cheadle Hulme, UK) coupled to an Elementar vario MICRO cube EA C/N analyser (Elementar Analysensysteme GmbH, Hanau, Germany). The following standards were used, in the order of appearance: quartz (blank) 10 mg, quartz (blank) 10 mg (again), HOS (high organic standard) 20 mg, USGS 25 (NH_4SO_4) 2.4 mg, cellulose 4.8 mg, CaCO3 16 mg, caffeine 4 mg, nitrogen standard 1 2.4 mg, nitrogen standard 2 2.4 mg, $CaCO_3$ 16 mg, pine needle 5-10 mg and HOS 20 mg. Every 15 samples, we weighed again 20 mg of HOS. The measurements were done in groups of maximum 64 samples, before which a set of standards was run each time.

2.8. Soil material

After removing the plants, the top 10 cm of soil within the sample area was mixed, sample was taken as randomly as possible and put in 100 ml plastic cups with screw-on lid. After this, the soil was dug out until there was a flat area at 20 cm depth equivalent to that on the surface, the soil was mixed at 20-30 cm depth and a sample was taken as randomly as possible in 100 ml plastic cups. All samples were immediately frozen for further analysis.

From the soil material, OC, total nitrogen (TN) and $\delta^{13}C$ ratio were measured via elemental analysis. For this, frozen samples had to be milled, then dried and weighed in tin boats. The amount of soil

sample weighed in each tin boat was between 50 and 100 g. These samples were ground and measured like the plant material samples.

For measuring pH and conductivity, 10 g of dry soil were suspended in 25 ml distilled water, the sample was properly shook and then measured on the following day. We used a conductivity measurer (Cond 340i (de-vice) and TetraCon® (electrode), Xylem Analytics Germany Sales GmbH & Co. KG, WTW, Weilheim, Germany) and pH measurer (inoLab® pH 720, Xylem Analytics Germany Sales GmbH & Co. KG, WTW, Weilheim, Germany).

Fatty acids (FAs) from phospholipids of the soil microbiota were extracted from the soil samples as a way of having an insight on the abundance and diversity of soil microbiota among the different soil treatments.

2.8.1. PLFAs

Phospholipids are the main components of all living cell membranes. They are constituted by a polar phosphorous group and two apolar FAs, all of which are linked together with a glycerol group (figure 2.6.) (Kandeler 2015). When in the cell membrane, which is a lipidic bilayer, the polar phosphorous groups face outwards (towards the outside and the inside of the cell) whereas the apolar FAs face inwards forming a barrier that cannot be trespassed by charged substances. The degree of unsaturation of the fatty acids affects the fluidity of the cell membranes, due to their spatial configuration. Thus, unsaturated fatty acids, those that present double bonds in the C chain, do not have a straight spatial configuration, establishing weaker interactions with the other fatty acids within the lipidic bilayer and, in doing so, increasing the fluidity of the membrane (Lodish *et al.* 2008).

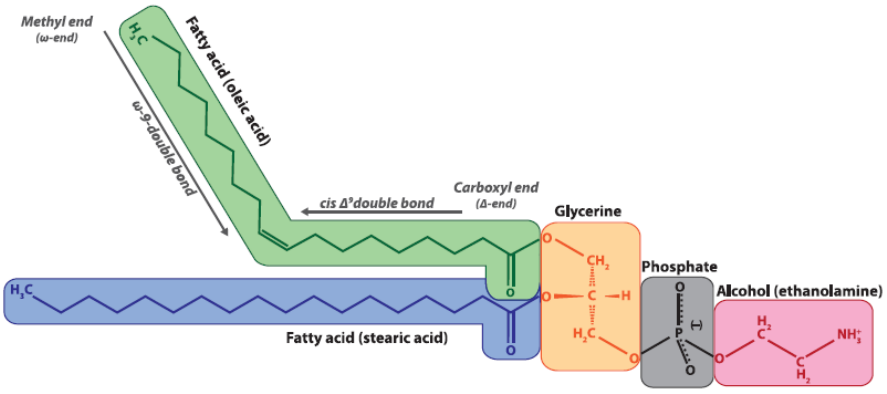

Figure 2.6. Phospholipid chemical structure (Kandeler 2015). The polar phosphorous group is composed of the phosphate (grey) and, in this case, alcohol (pink) groups shown above; and it faces out of the cell membrane. The glycerine group (yellow) links the phosphorous group to the fatty acids. The fatty acids may be saturated (blue) or unsaturated (green) influencing the lipidic bilayer´s fluidity.

Neutral lipids mainly refers to energy storage triglycerides. These have a similar structure as the membranes phospholipids but, instead of having one of the glycerol´s carbons forming a bond with the phosphorous group, it is linked to another fatty acid. In this way, energy storage triglycerides are constituted by a group of three fatty acids linked to a glycerol group (Lodish *et al.* 2008).

FAs are synthesised by organisms although heterotrophs can incorporate them directly from their diet. When synthesised, FAs can have a longer or a shorter C chain and present unsaturations at different positions depending of the path way of biosynthesis. This is why FAs can be classified according to their unsaturations in different omega (ω - end) groups (Kandeler 2015), for we count the number of C atoms from the terminal methyl group to the position of the unsaturation(s). PLFAs and NLFAs (FAs obtained from neutral lipids) that have been obtained from pure culture studies can be used as biomarkers of specific taxa (Kandeler 2015).

The PLFA pattern of soil organisms is a tool that has helped to open the "black-box" of soil microbiota and is a common method used to study microbial community structure (Ruess 2010, Frostegard, 2011, Kandeler 2015, Willers *et al.* 2015) for it offers a number of advantages when compared to others. It is a method that it is rapid, relatively cheaper than other methods, such as qPCR, and reproducible (Frostegard, 2011, Willers *et al.* 2015). What is most interesting is that the FAs present in a soil at a given moment provide information on the microbiota that is actually present in that soil at that given moment (Kandeler 2015). This is due to the rapid release and degradation through enzymatic reactions that FA experiment in the soil after cell death (Olson, 1999; Ruess and Chamberlain 2010; Frostegard 2011, Kandeler 2015, Willers *et al.* 2015).

In Table 2.3 the different PLFAs used as biomarkers in this study are presented, with the species they designate and references to the original citations. Willers *et al.* 2015 compiles information on recent studies that establish these PLFA biomarkers and others that are used.

Table 2.3. FA biomarkers analysed (adapted from Willers *et al.* 2015)

Fatty Acids	Lipid fraction	Designation	Original citations	Sample origin
Straight chain saturated FAs				
14:0	PLFA	Bacteria	Zelles, 1997	Pure cultures; soil
15:0	PLFA	Bacteria	Zelles, 1997	Pure cultures; soil
17:0	PLFA	Bacteria	Zelles, 1997	Pure cultures; soil
18:0	PLFA	Bacteria	Zelles, 1997	Pure cultures; soil
Cyclopropyl branched FAs				
Cy19:0	PLFA	Gram-negative bacteria	Wilkinson, 1988	Pure cultures; soil
10-Methyl branched saturated FAs				
10Me16:0	PLFA	Actinomycetes	Kroppenstedt, 1985; Vestal and White, 1989	Pure cultures; soil
Terminally branched saturated FAs - Anteiso				
a15:0	PLFA	Gram-positive bacteria	O´Leary and Wilkinson, 1988; Vestal and White, 1989	Pit mud; pure cultures; soil.
a17:0	PLFA			

- Iso				
i15:0	PLFA	Gram-positive	O´Leary and Wilkinson,	Pit mud; pure
i16:0	PLFA	bacteria	1988; Vestal and White,	cultures; soil.
i17:0	PLFA	(*idem*)	1989	(*idem*)
Monosaturated FAs				
16:1ω5c	PLFA	AMF	Pacovsky and Fuller, 1988; Olson et al. 1995	Plant roots, soil
16:1ω7c	PLFA	Gram-negative bacteria, Cyanobacteria, diatoms	Wilkinson, 1988; Ahlgren et al. 1992	Pure cultures, soil Fresh water microalgae, microbial mats, pond water
18:1ω7c	PLFA	Gram-negative bacteria, Cyanobacteria, diatoms	Wilkinson, 1988; Ahlgren et al. 1992	Pure cultures, soil Fresh water microalgae, microbial mats, pond water
18:1ω9c	PLFA	Fungi, Cyanobacteria, green algae	Vestal and White, 1989 Ahlgren et al. 1992	Pure cultures, soil Fresh water microalgae, microbial mats, pond water
Polyunsaturated FAs				
18:2ω6,9c	PLFA	Saprotrophic fungi	Federle, 1986, Stahl and Klug, 1996	Pure cultures, soil
		Cyanobacteria, diatoms	Ahlgren et al. 1992	Fresh water microalgae, microbial mats, pond water
18:3ω3,6,9c	PLFA	Fungi	Zelles, 1997	Biofilms, pure cultures, soil
		Cyanobacteria	Ahlgren et al. 1992	Freshwater, microalgae; microbial mats
20:4ω6,9,12,15c	PLFA	Cyanobacteria, diatoms, green algae	Volkman et al. 1989 Ahlgren et al. 1992	Freshwater, microalgae; microbial mats

2.8.2. PLFA extraction

In order to obtain the PLFAs from the soil microbiota, the FAs of interest had to be extracted from the cells of the microbiota in the soil samples. After this, the FAs had to be hydrolysed, methylated and

separated from their polar groups. After derivatisation the samples were ready to be measured with a gas chromatograph (Agilent Technologies 7890A GC system) coupled to an isotope analyser (Elementar IsoPrime 100 IRMS (IsoPrime Ltd, Cheadle Hulme, UK)). The method is based on the original protocol in Olson (1999) and was adapted by the laboratory team at the Institute of Soil Science of Leibniz University Hannover. PLFAs and NLFAs were both extracted. The extraction can be divided into three steps:

1. Extraction of the lipids from the soil samples.
    a. The frozen soil samples were ground in a ceramic mortar with liquid nitrogen. The day before starting, they were placed in a refrigerator so that the next day about 6 g of each soil sample could be weighed in 50 ml glass centrifuge tubes.
    b. First, the decided amount of PLFA and NLFA specific standards was added and then 20ml of Bligh and Dyer solution ($CHCl_3$:MeOH:Citrate buffer, 1:2:0.8) to extract the lipids present in the soil samples. Tubes are put on a horizontal shaker (HS 501 digital, IKA®-Werke, GmbH & Co. KG, Staufen, Germany) at 225 rpm for 1h, then 15 min in ultra-sonification bath (Sonorex, BANDELIN electronic GmbH & Co. KG, Berlin, Germany) and finally back on the horizontal shaker for another hour at 225 rpm. Next, they were centrifuged at 3000 g for 12 min and 7 ºC (Cryofuge 6000, Heraeus Holding GmbH, Hanau, Germany). Supernatant was then transferred into a 250 ml separating glass funnel with Teflon taps.
    c. A second extraction was done by adding 10 ml of Bligh and Dyer solution again, shaking on the horizontal shaker as before but for 30 minutes, centrifuging once again as described above and finally adding the supernatant to the separating funnel with the previous supernatant for the respective sample.
    d. To establish a two phase solution, that separates lipids from water soluble substances (like carbohidrates and proteins), 7.5 ml chloroform and 7.5 ml citrate buffer were added. The funnels were shaken intensely and then placed on a circular shaker (KS 260 basic, IKA®-Werke, GmbH & Co. KG, Staufen, Germany) with a mount for separating funnels at 200 rpm for 15 min. Then they were placed back on their brackets and waited between 1 and 2 hours for the phase separation.
    e. When the two phases were clearly separated, the lower phase was introduced into 25 ml copped bottom flasks. 7.5 ml of chloroform were added to the funnels, they were shaken intensely and put back on the circular shaker with mount, like before. Funnels were then placed back in the brackets until the following day to combine the lower phases in the copped bottom flask. This lower phase contains the lipids immerse in the chloroform.
2. Lipid fractionation with solid phase extraction (SPE) silica columns dependent on polarity.
    a. The lipidic phase in the copped bottom flasks is concentrated on a rotation evaporator (Laborota 4000 efficient, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at 35º C to less than 0.5 ml. Automatic program set on the rotation evaporator was as follows: 0” at ambient pressure, 30” decrease to 700 mbar, 2’ 32” decrease to 600 mbar, 3’ 32” decrease to 500 mbar, 4’ 30” decrease to 350 mbar, 5’ 30” decrease to 200 mbar, 6’ 30” maintain at 200 mbar.
    b. For fractionation silica gel columns were used. The columns were baker glass columns with an inner diameter of 10 mm, that contained 1.5 cm of activated silica gel particle size 0.063 – 0.200 mm (silica gel Merck, silica 60) between two glass fibre filters. The activated silica was always kept immersed in chloroform to avoid

deactivating it. At the bottom of the baker columns, there were stainless steel or Teflon Luer taps to regulate the flow through the columns.

c. The lipidic concentrate was added to the silica columns for first fractionation, rinsing the copped bottom flask three times with chloroform. Then 2.5 ml chloroform were added to the column twice while letting the NLFA elute slowly (2 drops/s) into clean 50 ml copped bottom flasks. Their volume was reduced on the rotation evaporator at 35 ºC, with the same automatic program as described previously. The "NLFA concentrates" were then added to 5 ml reactivials (rinsing the copped bottom flasks three times with chloroform), where they were dried on a gentle nitrogen stream. Once dry, they were closed with a Teflon septa lid and stored in a freezer at -20 ºC.
d. When the previous elution finished, 20 ml of acetone were added to elute the glycolipids slowly (2 drops/s); which were discarded.
e. Finally, 20 ml of methanol were added twice to elute the PLFAs slowly (2 drops/s) into clean 50 ml copped bottom flasks. The volume was reduced on the rotation evaporator at 35 ºC, with a different program: 0" reduce to 800, 1' decrease to 500 mbar, 2' 30" decrease to 300 mbar, 3' 30" decrease to 150 mbar, 8' 30" maintained at 150 mbar. The "PLFA concentrates" were transferred to 5 ml reactivials (rinsing the copped flasks three times with methanol) and we dried the samples on a gentle nitrogen stream. Once dry, they were closed with a Teflon septa lid and stored in a freezer at -20 ºC.

3. Derivatisation
    a. Hydrolysation. First, 0.5 ml of 0.5 M NaOH in MeOH were added to the PLFA samples in the 5 ml reactivials and they were put in the ultra-sonification bath for about 10 min. The reactivials were placed on a derivatisation block at 100 ºC for 10 min, checking they were properly closed.
    b. Methylation. Once removed from the derivatisation block and after waiting until they had cooled down, 0.75 ml of 12.5 M BF_3 in methanol were added. The vials were properly closed and they were placed back on the derivatisation block at 80 ºC for 15 min. At this stage the fatty acid methyl esters were still linked to their head groups.
    c. Separation from the head groups. After adding 1 ml of saturated NaCl solution (for hydrolysation of BF_3, to reduce its toxicity) 2 ml of hexane were added into each vial and they were shaken on an automatic shaker (Multi Reax, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at maximum strength for 3 min. Only the upper apolar phase that contains the fatty acid methyl esthers was removed and then the process was repeated adding again 2 ml of hexane. After combining the hexane in a new reactivial, the samples were dried under a gentle nitrogen stream.
4. Preparation for measurement
    a. 30 µl of the fatty acid methyl ester (FAME) standard chosen solved in toluol (13:0, 1 µg/µl) was added in each vial together with another 170 µl of toluol. Vials were shaken on vortex mixer and solved in ultra-sonification bath for 10 min.
    b. Solution was transferred to a 100 µl inlet inside a 1.5 ml GC-Autosampler-Vial, lids were crimped on them and they were kept in a freezer at -20 ºC until measurement.

The measurements were done with a gas-chromatograph (7890A GC System, Agilent Technologies, Santa Clara, United States) coupled with isotope probe (to detect presence and abundance of $\delta^{13}C$). A capillary column HP-5 (Agilent Technologies; length 60 m, 0.32 mm I.D., 0.25 µm film thickness) was used for separating the single components. A volume of 1 µl was injected into the injector running at a temperature of 250 °C in splittless mode. The capillary column was connected to the infrared-

mass-spectrometer (IRMS) via a combustion interface for transferring the FAME molecules to CO_2 at 850 °C. Water was removed after this using a nafion tube and reference gas pulses of CO_2 where used for online calibration during the measurement. As a result of this, a graph with various peaks with varying heights and widths is obtained. Each of those peaks corresponds to the combustion of a molecule, mainly the FAME molecules that have been isolated in the extraction process described.

24 samples were prepared per week; doing extraction, lipid fractionation and derivatisation on separate days. Samples for day 0, day 1, day 11 and day 21 were prepared and analysed. As well as the samples, multistandard samples had to be prepared with increasing concentrations. These were extremely important because they allow the calculation of the other samples´ concentrations, together with the known quantity standards that are added at different stages throughout the extraction process to estimate recovery rate.

The different FAME peaks obtained from the gas-chromatography were identified by comparing them with the multistandard samples, by using IonOS software (IonOS 3.0.7.5327, Isoprime Ltd.). After having recognised and assigned the correct FAMEs to each peak, reports were created with the software to interpret raw data and estimate amount of PLFAs and NLFAs in the soil of each plot sampled.

2.9 Calculations

Net ecosystem production (NEP)

NEP refers to the net organic carbon production via plant photosynthetic activity minus the C lost due to plant respiration and respiration from soil microorganisms that feed on its structures or exudates (Kirschbaum *et al.*, 2001). It can be calculated as follows:

$$\mathrm{NEP} = \mathrm{NPP} - \mathrm{Rs}$$

where NPP would be Net Primary Production, which refers to C fixed via photosynthesis minus the C lost via plant respiration, and Rs accounts for the C loss from soil microorganism respiration.

Excess ^{13}C in different compartments

The isotopic value was expressed as the variation relative to the Vienna Pee Dee Belemnite (VPDB) standard (delta (δ) notation used). Therefore, the amount of ^{13}C in the sample was calculated in atom% (% of the total C atoms) according to the following formula (Ruehr *et al.*, 2009):

$$atom\% = \frac{100 \times R_{VPDB} \times (\frac{\delta^{13}C}{100} + 1)}{1 + R_{VPDB} \times (\frac{\delta^{13}C}{100} + 1)}$$

where $R_{VPDB} = 0.0111802$ is the standard value for isotope ratio (Hut, 1987).

- Excess ^{13}C in plant roots and shoots

Excess ^{13}C refers to the amount of ^{13}C there is in a plant root or shoot sample after labelling when compared a sample of the same characteristics before labelling. The excess of ^{13}C in the plant (in mg ^{13}C m^{-2}) was calculated by the following:

$$Excess^{13}C_{Plant} = \frac{atom\%_P - atom\%_C}{100} \times \frac{DW \times OC(\%)}{100}$$

where atom%$_P$ is the ^{13}C value in the labelled plant and atom%$_C$ the ^{13}C value in the unlabelled plant control, DW is the dry weight of plant material (g m^{-2}) and OC the organic carbon content (in %). More precisely, excess ^{13}C in plant roots and shoots is the total amount of ^{13}C added by the pulse labelling to the plant´s mass per square meter.

- Excess ^{13}C at different soil depths

The excess ^{13}C at different soil depths was calculated by the following equation per volume of soil air (µg C dm^{-3}):

$$Excess^{13}C_{Soil} = \frac{atom\%_S - atom\%_C}{100} \times \frac{C(\mu g)}{V\ (dm^{-3})}$$

Where C is the concentration of CO_2-C in soil air and V the volume of the soil air suction cups. So, excess ^{13}C at different soil depths refers to the total amount of ^{13}C added by the pulse labelling to CO_2 in the soil air.

- Mean residence time (MRT)

MRT, in this case, is the time the ^{13}C remains in a given soil. The MRT of the ^{13}C in soil was calculated by fitting the excess $^{13}C_{soil}$ value to a first order exponential decay function to the values of the three replicates:

$$C(t) = C_0 \times e^{-kt}$$

with k as decay rate constant and, C(t) is the quantity of ^{13}C after labelling at time after labelling (t), and C_0 the initial quantity of excess ^{13}C. The MRT then obtained from the reciprocal function of k:

$$MRT = \frac{1}{k}$$

- Excess ^{13}C in soil CO_2 fluxes

Regarding the ^{13}C content in the CO_2, we could calculate the ^{13}C efflux from the soil using the Keeling plot method (according to Pataki *et al.*, 2003):

$$\delta^{13}C_a = c_b(\delta^{13}C_b - \delta^{13}C_S) \times \left(\frac{1}{c_a}\right) + \delta^{13}C_s$$

Where $\delta^{13}C_a$ is the ^{13}C isotope ratio in atmospheric CO_2 concentration, $\delta^{13}C_b$ is the ^{13}C isotope ratio for background CO_2 concentration, and $\delta^{13}C_S$ is the integrated value of the CO_2 in the soil.

Thereafter, the excess ^{13}C in soil CO_2 flux was calculated using the following equation:

$$Excess^{13}C_{Flux} = \frac{atom\%_S - atom\%_C}{100} \times F$$

Where the excess $^{13}C_{Flux}$ is given in µg C m^{-2} h^{-1}, atom%$_S$ is the ^{13}C value in the soil CO_2 efflux on labelled plots and atom%$_C$ the ^{13}C value in the unlabelled control, and F is the CO_2 flux from the measurement day.

2.10. Statistical Analyses

Differences between treatments (e.g. catch crop variant) have been evaluated using linear mixed effects models (LMM) in order to account for the repeated measurements at different dates and the hierarchical nesting of a given sample per plot (Bates *et al.*, 2014).

For differences in excess ^{13}C rates, catch crop variants have been set as fixed effects while sampling time and plot were set as random variables in order to account for the differences between treatments at individual time-points. Residuals were checked for normality and log transformation to the data was applied when needed.

Non metric multidimensional scaling (NMDS) was used to visualize the differences in microbial community between sampling points and treatments. NMDS is an ordination method that uses distance based matrixes as and input. The Bray-Curtis distance matrix algorithm was applied after applying a rank index function (R vegan package) and a stress-plot was used to check the goodness of fit

All statistical analyses were performed with R version 3.4.0 (R Core Team, 2017).

3. Results

3.1. Net ecosystem production (NEP)

By comparing the NEP of the different catch crop treatments, the efficiency of C allocation of the different catch crop treatments can be observed. In figure 3.1. the calculated NEP values for the different treatments is shown. The negative NEP for the fallow provides information on the C release due exclusively to microbial respiration from the soils, for there was no plant cover. There were positive NEP values for the catch crop treatments, as was expected. We can see in figure 3.1. that the increase in the NEP values of the different treatments increases alongside species diversity, showing that the C assimilation of the TerraLife mix is three times higher than that of the Mustard.

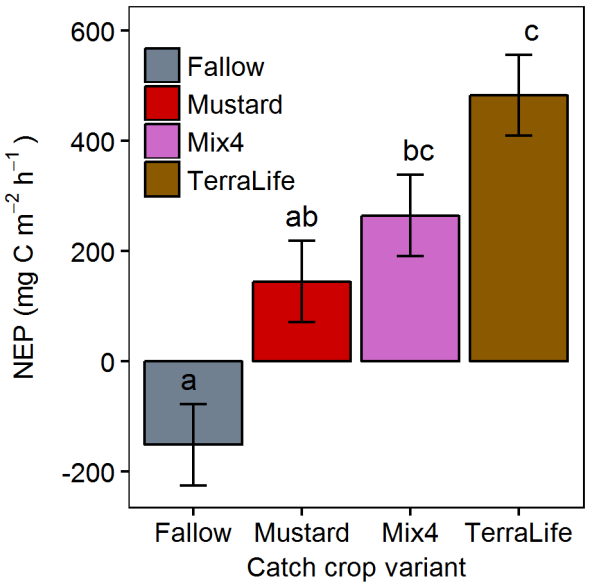

Figure 3.1. NEP values of the different catch crop treatments and the fallow soil. NEP was calculated on the field plot base. Small letters denote statistic differences evaluated by linear mixed effects models with "date" as random variable.

3.2. Excess ^{13}C in different compartments

3.2.1. Excess ^{13}C in plant roots and shoots

The ammount of excess ^{13}C in the plants (Figure 3.2.) did not correspond to the initial amount of ^{13}C added (252 mg ^{13}C m^{-2}). This was due to the disproportion in biomass between leafs and stems. Leafs perform the photosynthetic uptake and therefore, have a higher ^{13}C signature than stems which are the transporters of assimilation products. So, the higher biomass of stems diluted the total biomass (ca. ratio leaf to stem 1:3) and the excess of ^{13}C is slightly overestimated. However, it does not compromise our approach.

Figure 3.2. shows a constant decrease of excess ^{13}C in roots and shoots of all the treatments over time. Shoots have more excess ^{13}C than roots, being an order of magnitude higher. The TerraLife roots showed significantly higher excess ^{13}C than the Mustard or Mix4. In the TerraLife roots, the excess ^{13}C decreased from 57 mg m^{-2} on the day after labelling, to 37 mg m^{-2} on the last sampling day. In the shoots, the Mix4 and the TerraLife presented similar values and evolution over time of the excess ^{13}C content, both being significantly higher than the Mustard. The values for the TerraLife decreased from 810 mg m^{-2} on the day after labelling to 570 mg m^{-2} on the last sampling day, and for the Mustard, from 570 mg m^{-2} to 320 mg m^{-2}.

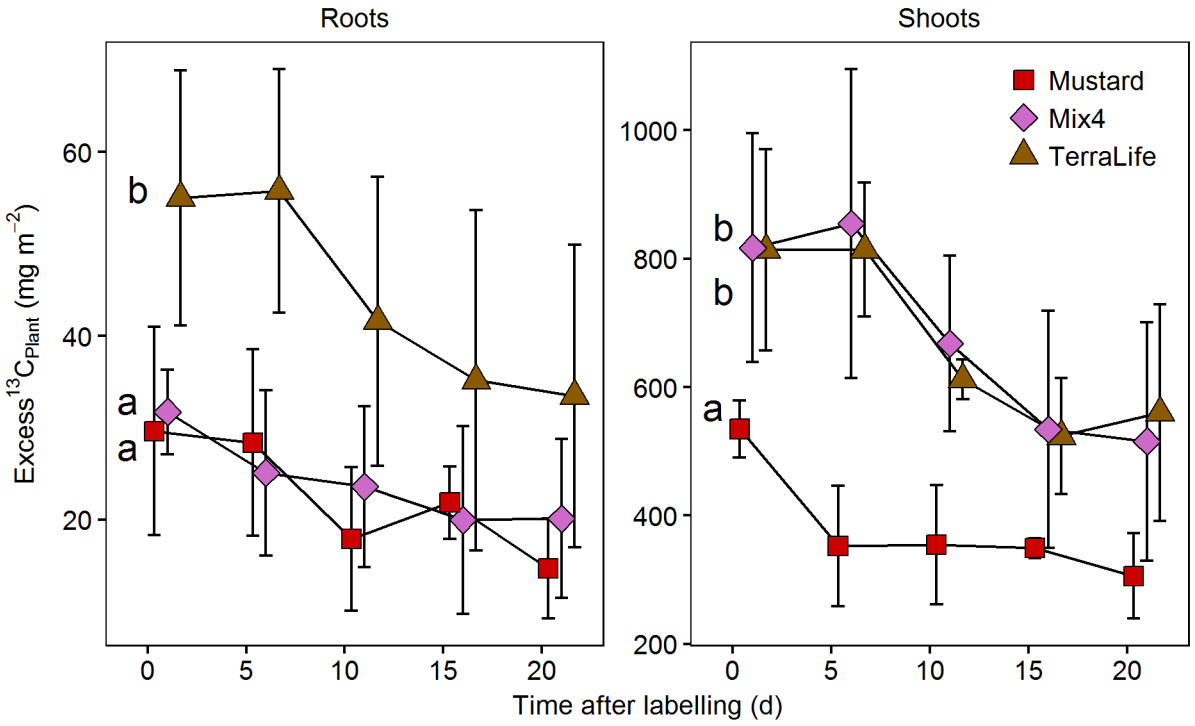

Figure 3.2. Excess ^{13}C in the different catch crop treatments´ plants´ shoots and roots after labelling. Differences between treatments were analysed with LMM using time and plot as random variable. Small letters denote significant differences at $p < 0.01$. Note, values on the x-axes are presented as factors in order to avoid overlapping of symbols.

3.2.2. Excess ^{13}C at different soil depths

The average $\delta^{13}C$ values in soil air were highest the day after labelling at 10 cm and 20 cm depth for all the labelled catch crop treatments (figure 3.3.). However, on the next sampling day (five days later) the values a much lower in all cases. The fallow (unlabelled control) was used as blank.

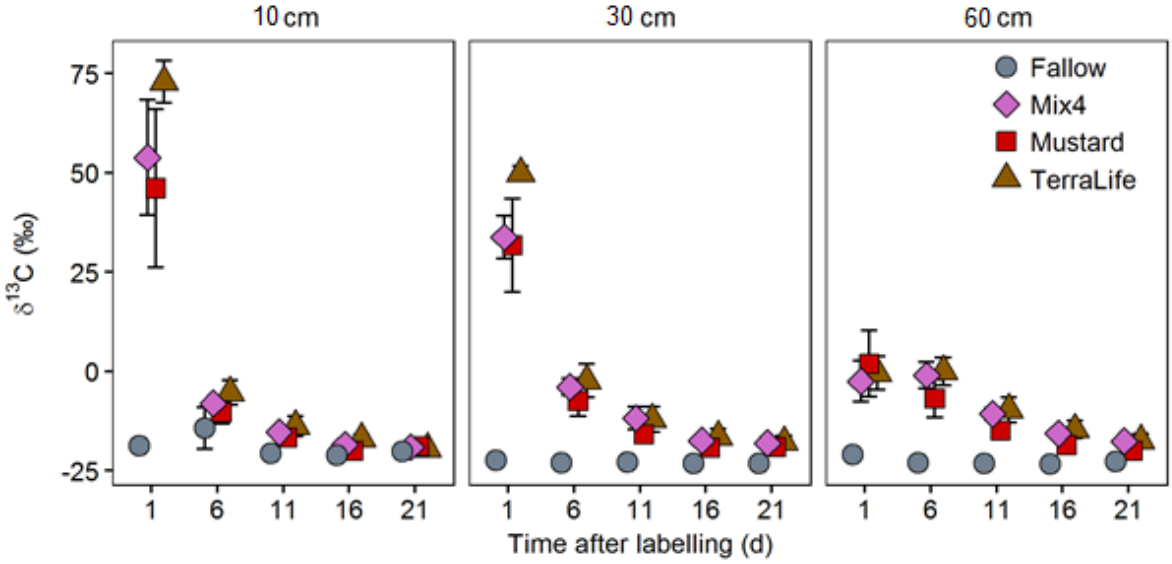

Figure 3.3. Average δ13C values (±SE) in soil air sampled after labelling from 10, 20, and 60 cm soil depth. Each dot represents the mean of three measurements on individual plots. The fallow plot represents the unlabelled control. Note, errors are partially smaller than symbols in some cases and values on the x-axes are presented as factors in order to avoid overlapping of symbols.

Figure 3.4 shows the fitted exponential decay function of ^{13}C and the MRT for the three depth increments. MRT increases with soil depth and presents significant differences at 60 cm depth for TerraLife and Mix4 in comparison to Mustard.

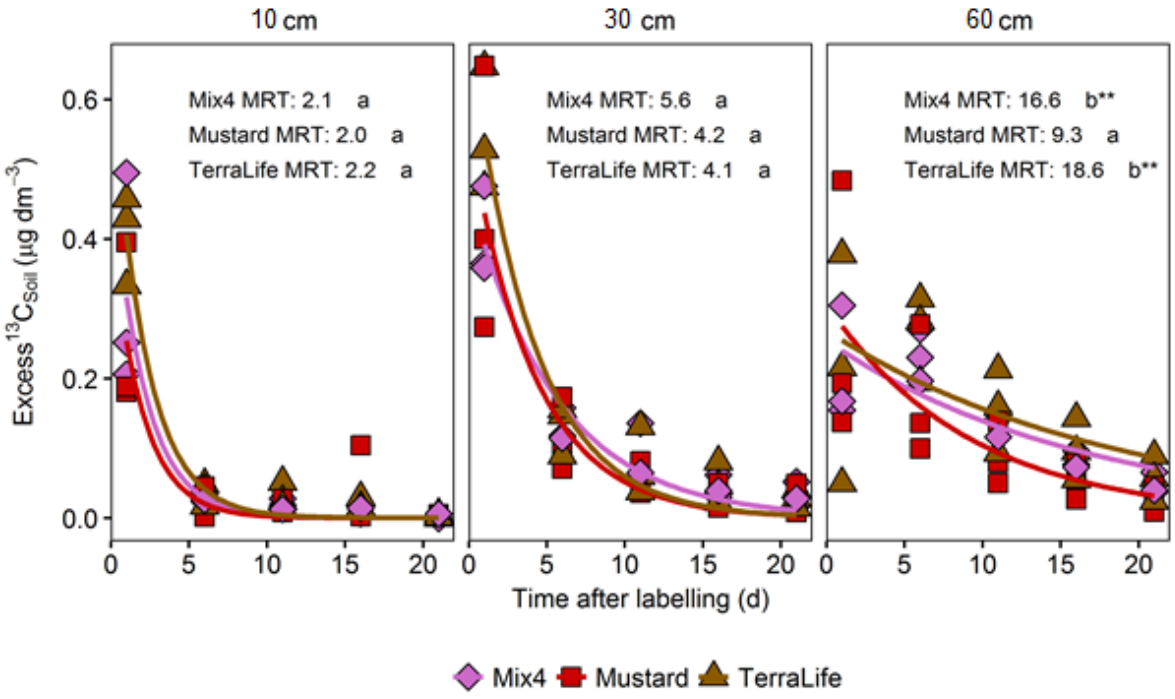

Figure 3.4. Evolution of excess ^{13}C-CO_2 in soil air of different depths. The MRT is given in days. Differences between the curves of treatments were evaluated with LMMs with "Time after labelling" as random effect. Small letters denote significantly different statistical groups (**, $p < 0.01$).

3.2.3. Excess ^{13}C in soil CO_2 fluxes

The excess ^{13}C in CO_2 from soil flux was significantly higher for the TerraLife soils than for the Mustard and the Mix4. The TerraLife presented a ^{13}C from CO_2 soil flux MRT of 2.9, whereas for the Mustard and the Mix4 the values were 2.6 and 2.3 respectively. The evolution over time is presented in figure 3.5.

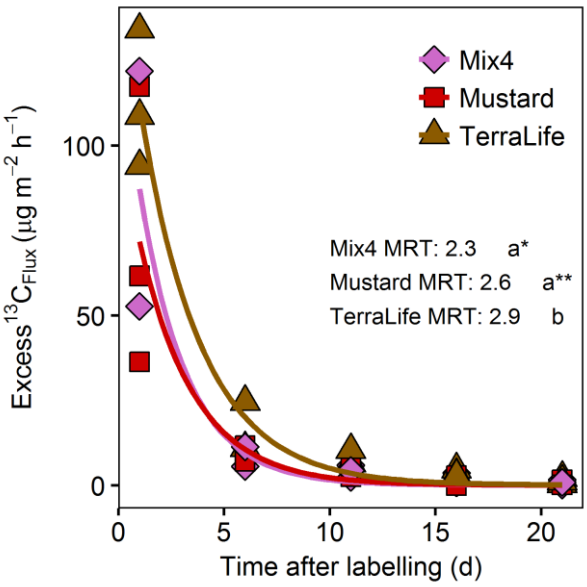

Figure 3.5. Evolution of excess ^{13}C-CO_2 in soil respiration fluxes after labelling. The mean residence time (MRT), the time of the ^{13}C remaining in the different soil layers, is given in days. Differences between the fitted curves were evaluated with LMMs with "Time after labelling" as random effects. Small letters denote significantly different statistical groups (*, $p < 0.05$; **, $p < 0.01$).

3.3. PLFA analyses

PLFA results in figures 3.6. to 3.12. are presented as amount of FA in nmol g^{-1} of soil (in the y axis), for each treatment with a different colour and symbol, per sampling day (in the x axis). The x axis of the graphs present the sampling day as a factor variable in order to avoid overplotting of symbols. Therefore, the distances between the time intervals are not according to the real time scale.

Total PLFA, for a given treatment, refers to the mean value per day after the plot was labelled, of the amount of PLFAs obtained from the sum of the measured PLFAs (presented in table 2.3) of each sample. Total PLFA data are presented in figure 3.6.

In the upper 10 cm of soil, total PLFA was significantly higher in the TerraLife soil than in the fallow soil. The PLFAs in the TerraLife soil also show a tendency to be higher than Mix4 and Mustard but this was not statistically significant. From 20-30 cm depth, there are no statistical differences among treatments in the amount of total PLFA extracted from the soil. However, for the TerraLife, it tends to decrease towards the last day; whereas in the first 10 cm of soil it tends to increase at that same time point.

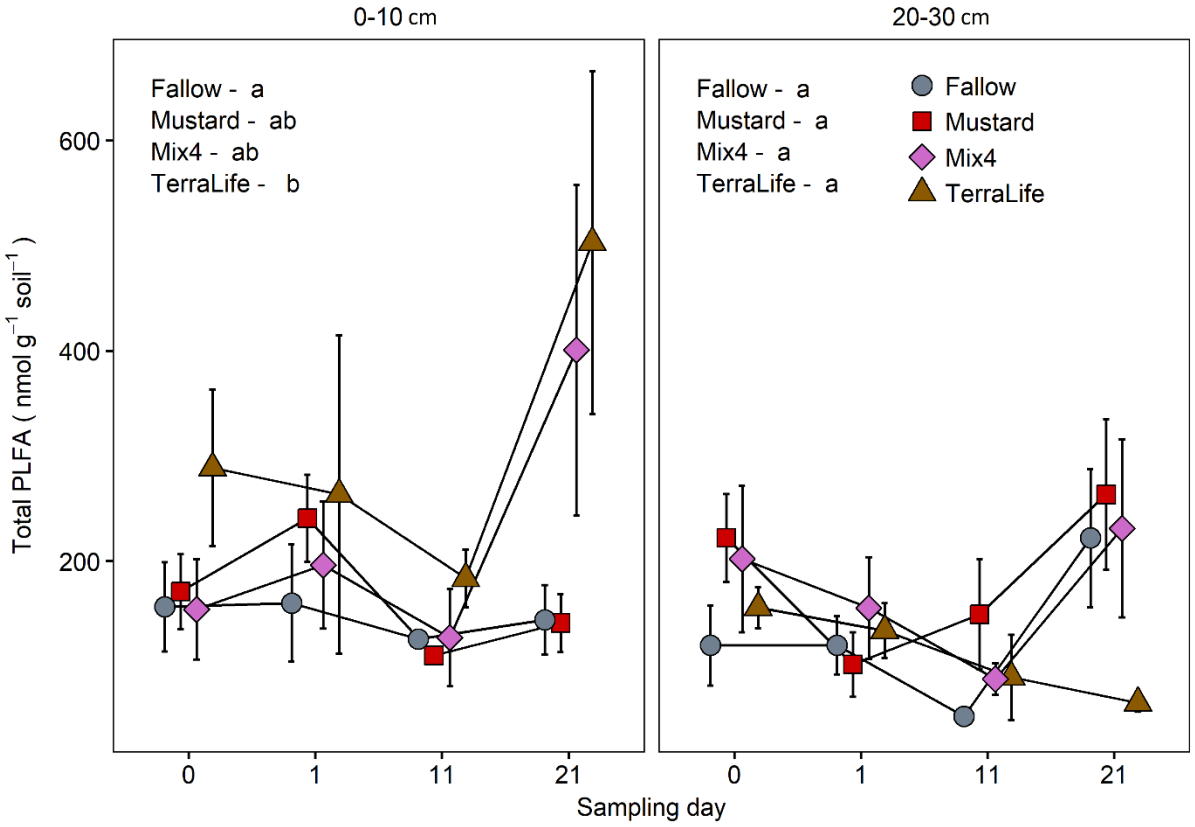

Figure 3.6. Total PLFA per each treatment group and sampling date from surface to 10 cm depth (left) and 20 - 30 cm depth (right). Note, values on the x-axes are presented as factors in order to avoid overlapping of symbols.

Fungal PLFA, for a given treatment, refers to the mean value per day after labelling, of the amount of PLFAs of the considered fungal PLFAs in table 2.3., in each sample. The fungal PLFAs considered are 18:2ω6,9c and 18:3ω3,6,9c, which are exclusive to fungi. Fungal PLFA data is presented in figure 3.7.

In the upper most 10 cm of soil, fungal PLFAs were significantly higher in the TerraLife than in the Fallow soil or in the Mustard's (Figure 3.7.). The Mix4 soil also tends to be higher throughout the experiment than that of the Fallow and the Mustard but it is not significantly different. There was significantly less fungal PLFAs than near the surface for all treatments with values that were not higher than 16 nmol g^{-1} $soil^{-1}$. No statistical differences among the treatments were found at 20 – 30 cm depth and the different treatments present a similar pattern throughout the sampling days; decreasing from day zero to day 11 (3^{rd} day) and increasing on day 21 (5^{th} day), except for the TerraLife, that keeps decreasing.

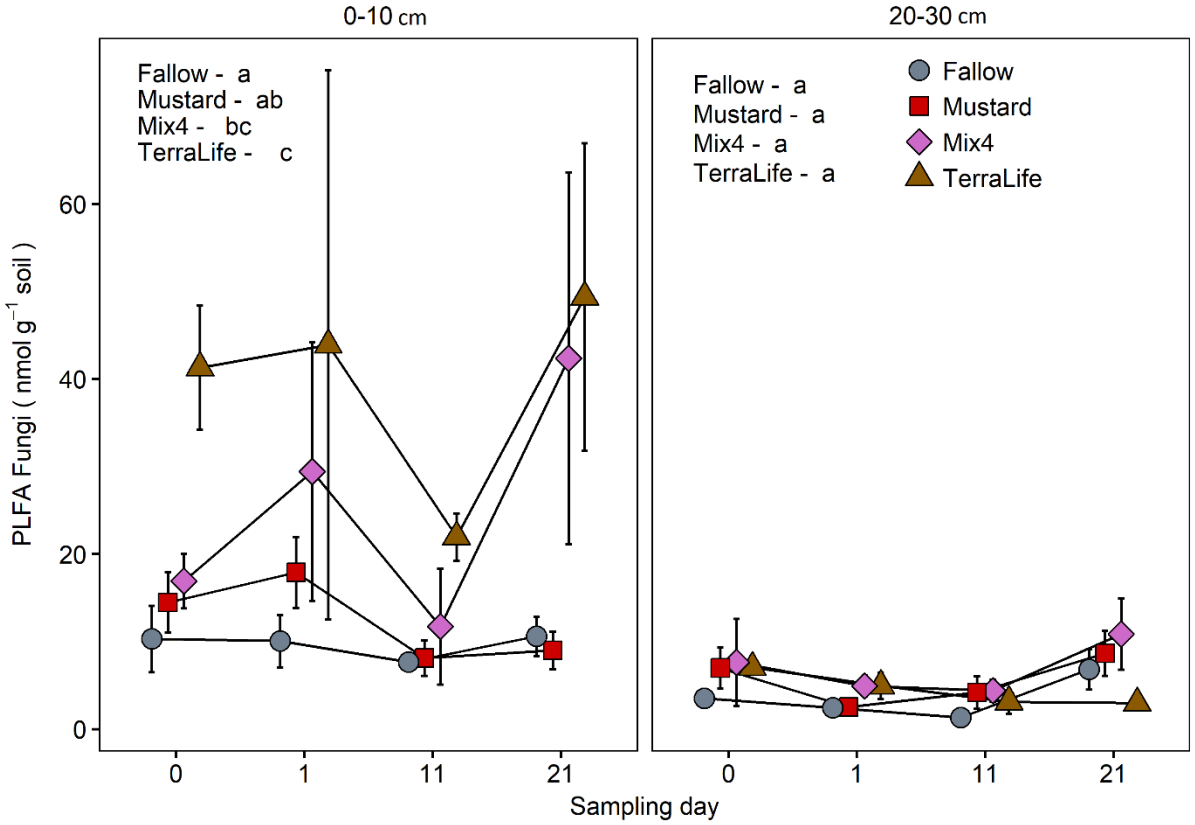

Figure 3.7. Sum of the considered fungal PLFA per each treatment group and sampling date from surface to 10 cm depth (left) and 20 - 30 cm depth (right). Note, values on the x-axes are presented as factors in order to avoid overlapping of symbols.

Bacterial PLFA, for a given treatment, refers to the mean value per day after labelling, of the amount of PLFAs of the considered bacterial PLFAs in table 2.3., in each sample. These bacterial PLFAs are 14:0, i15:0, a15:0, 15:0, i16:0, 16:1ω7c, i17:0, a17:0, 17:0, 18:0 and Cy19:0. Bacterial PLFA data is presented in figure 3.8.

In the first 10 cm depth there weren't any statistically significant differences, although in Mix4 soil and TerraLife soil bacterial PLFA increased noticeably on the last sampling day. From 20 to 30 cm, there were no significant differences either but we could observe a noticeable decrease of TerraLife bacterial PLFAs towards the last sampling day.

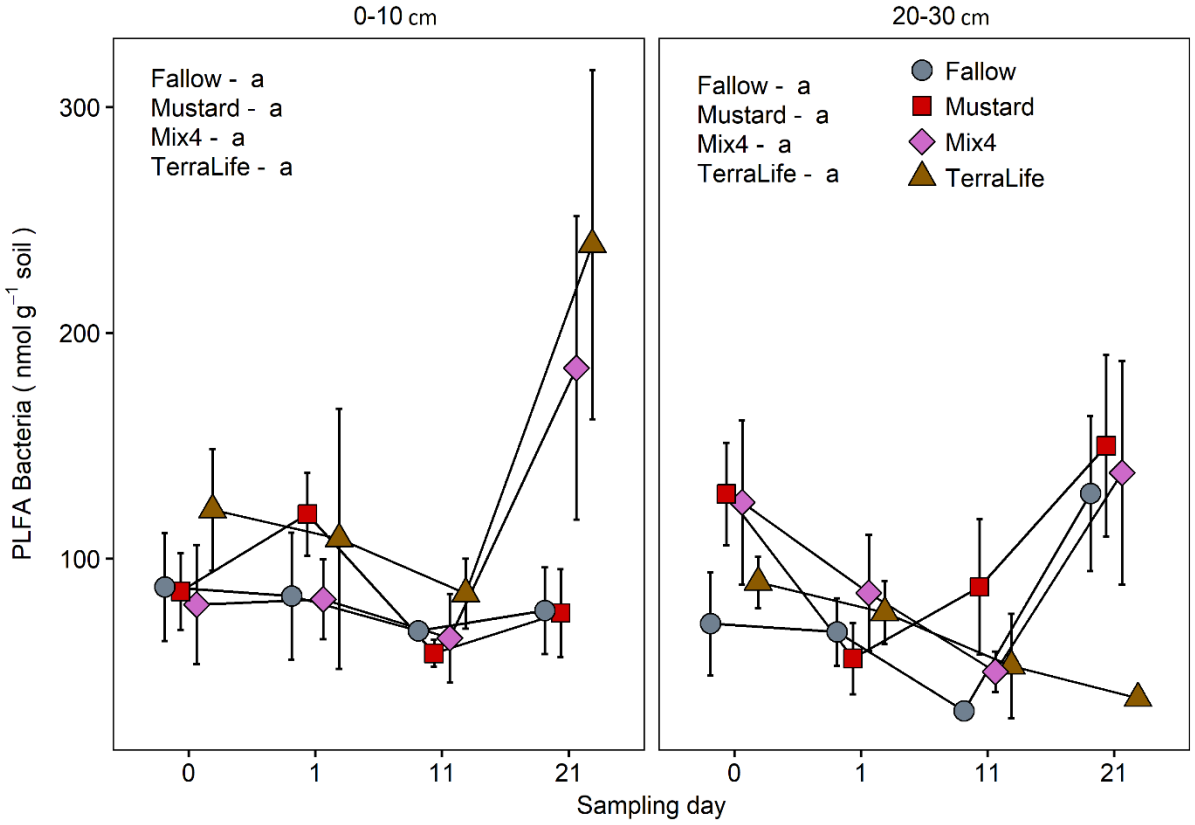

Figure 3.8. Sum of the considered bacterial PLFA per each treatment group for and sampling date from surface to 10 cm depth (left) and 20 - 30 cm depth (right). Note, values on the x-axes are presented as factors in order to avoid overlapping of symbols.

Fungi/Bacteria ratio refers to the relative amount of fungi over bacteria present in the soil, based on the considered fungal and bacterial PLFAs mentioned above. Fungi/Bacteria ratio is presented in figure 3.9.

When the Fungi/Bacteria ratio is observed regardless of soil depth , the TerraLife treatment's Fungi/Bacteria ratio is higher than that of the fallow soil. On days 0, 1 and 11, the Fungi/Bacteria ratio tends to increase with plant species diversity. Only on the last sampling day the Fallow's ratio has a slightly higher average value than the Mustard's, and so does the Mix4's when compared to the TerraLife's ratio value.

Regarding soil depth (figure 3.9), in the first 10 cm of soil, the Mix4 and TerraLife present significantly higher values of Fungi/Bacteria PLFA ratio than the Fallow and Mustard. The TerraLife values tends to decrease towards the end of the sampling days at this depth. At 20 – 30 cm soil depth, the Fungi/Bacteria ratio is much lower barely having values above 0.1.

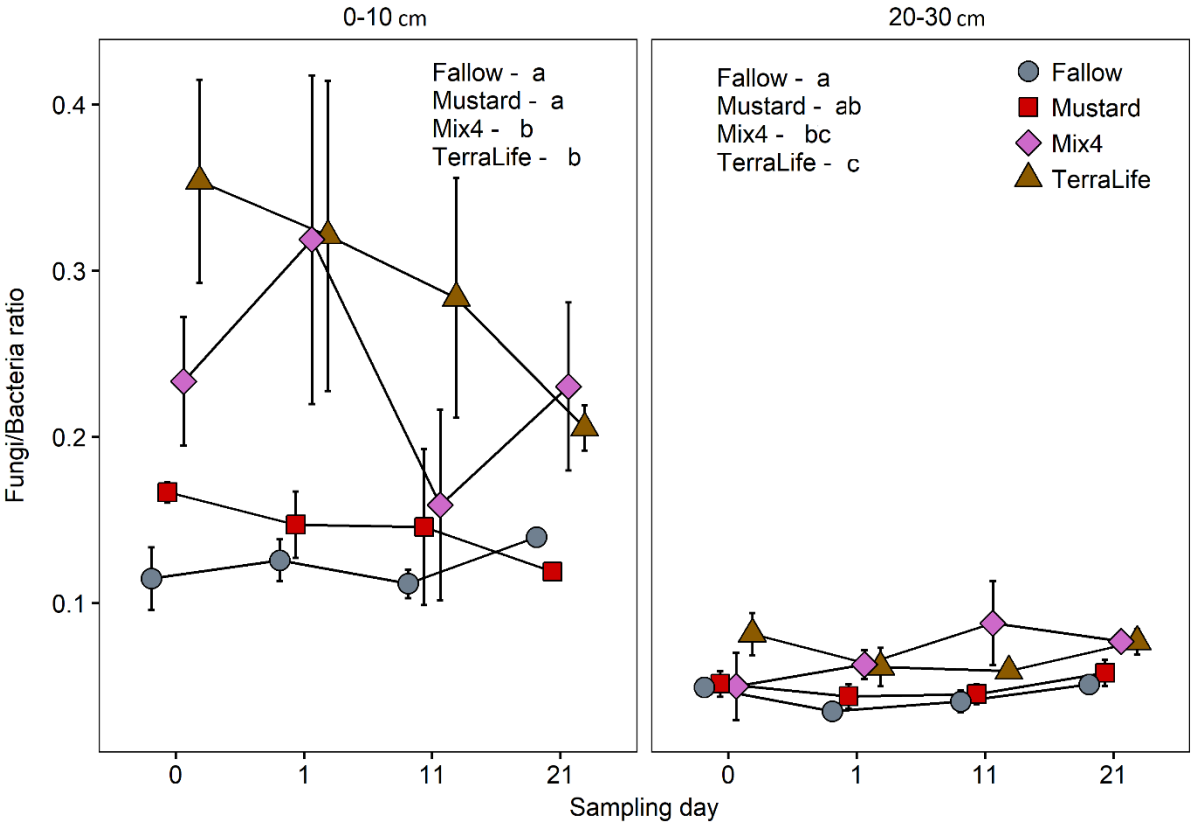

Figure 3.9. Fungi/Bacteria ratio per each treatment group and sampling date from surface to 10 cm depth (left) and 20 - 30 cm depth (right). Note, values on the x-axes are presented as factors in order to avoid overlapping of symbols.

To evaluate the gram-negative bacteria, PLFA Cy19:0 is considered as a biomarker for this group. Results are presented in figure 3.10., where a tendency of higher abundance of this PLFA in the TerraLife soil at 0 – 10 cm depth can be observed, increasing noticeably on the last sampling day. At 20-30 cm, the opposite happens; PLFA Cy19:0 occurrence decreases on the last sampling day. There are no statistical differences at this depth among treatments.

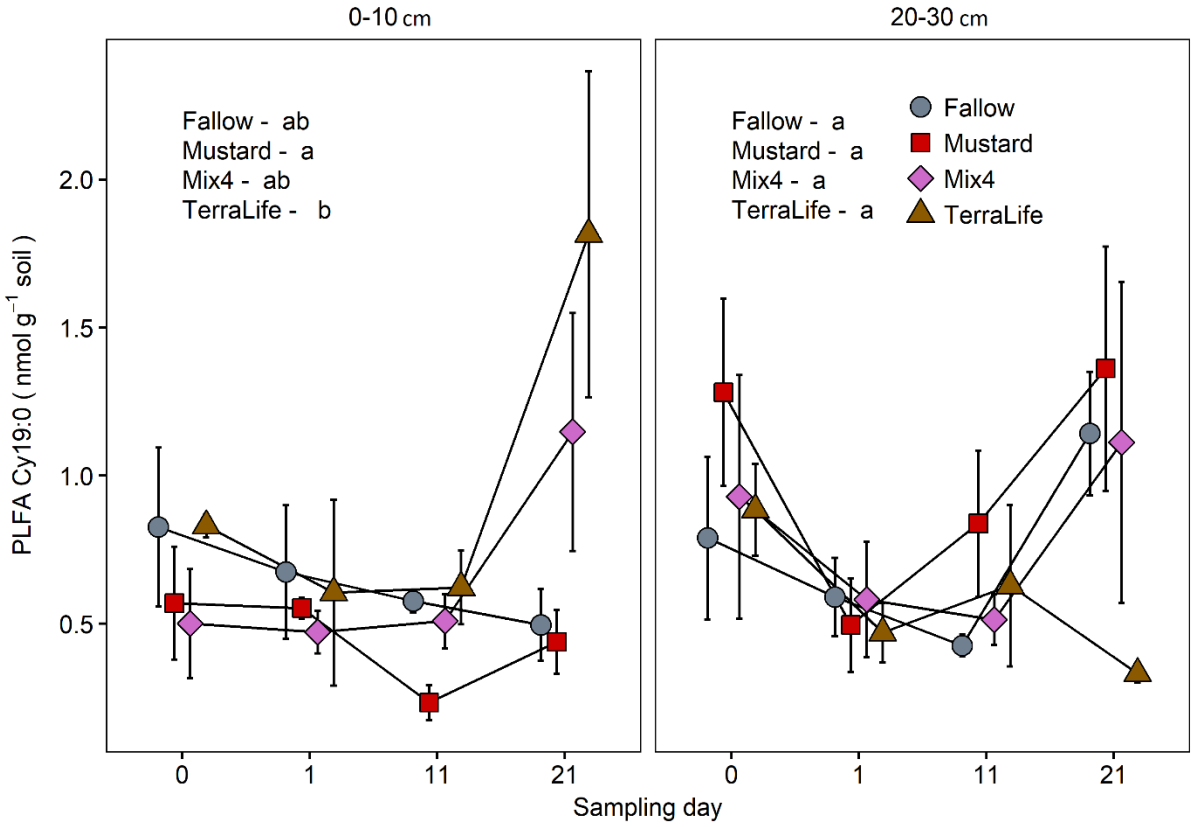

Figure 3.10. PLFA Cy19:0 presence as Gram-negative bacteria biomarker ratio per each treatment group and sampling date from surface to 10 cm depth (left) and 20 - 30 cm depth (right). Note, values on the x-axes are presented as factors in order to avoid overlapping of symbols.

PLFA 18:1ω7c was used as biomarker of AMF presence in the soil samples, and results are presented in figure 3.11. There were no statistical differences in its occurrence at both depths, although there is a clear increase tendency on the last sampling day for the TerraLife and the Mustard's soil in samples at 0 – 10 cm depth. In samples at 20 – 30 cm depth, PLFA 18:1ω7c in the TerraLife's soil tends to decrease on the last sampling day.

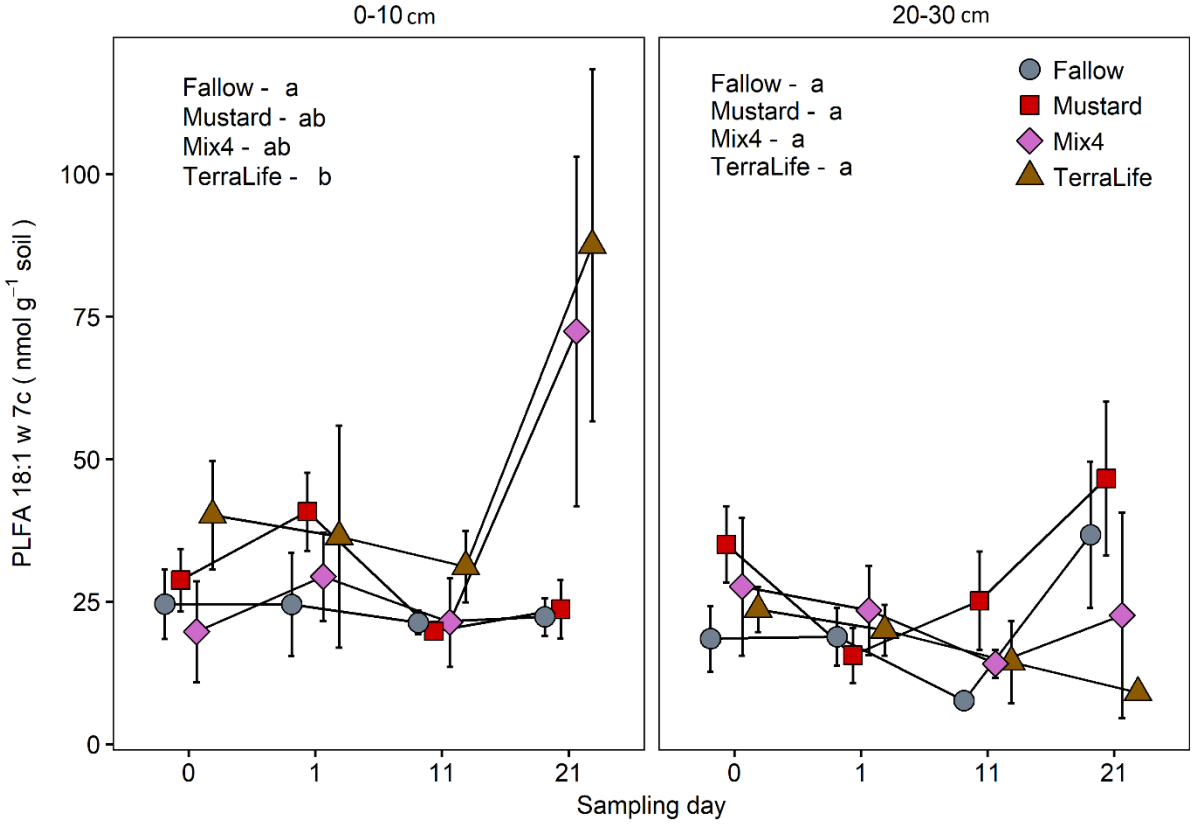

Figure 3.11. PLFA 18:1ω7c presence as AMF biomarker ratio per each treatment group for and sampling date from surface to 10 cm depth (left) and 20 - 30 cm depth (right). Note, values on the x-axes are presented as factors in order to avoid overlapping of symbols.

The figure 3.12., presents the PLFA analysis using the NMDS ordination method. This method presents relative PLFA contents per soil sample (symbols) and date of harvest after labelling (colours). The closer each coloured symbol is to the reference points, presented as black dots, the higher is the relative amount of the PLFA in that sample. The graph showed clear difference of the microbial community between the sampling days (colours of the same date cluster together). We did, however not find a clear pattern of microbial community change between catch crop treatments.

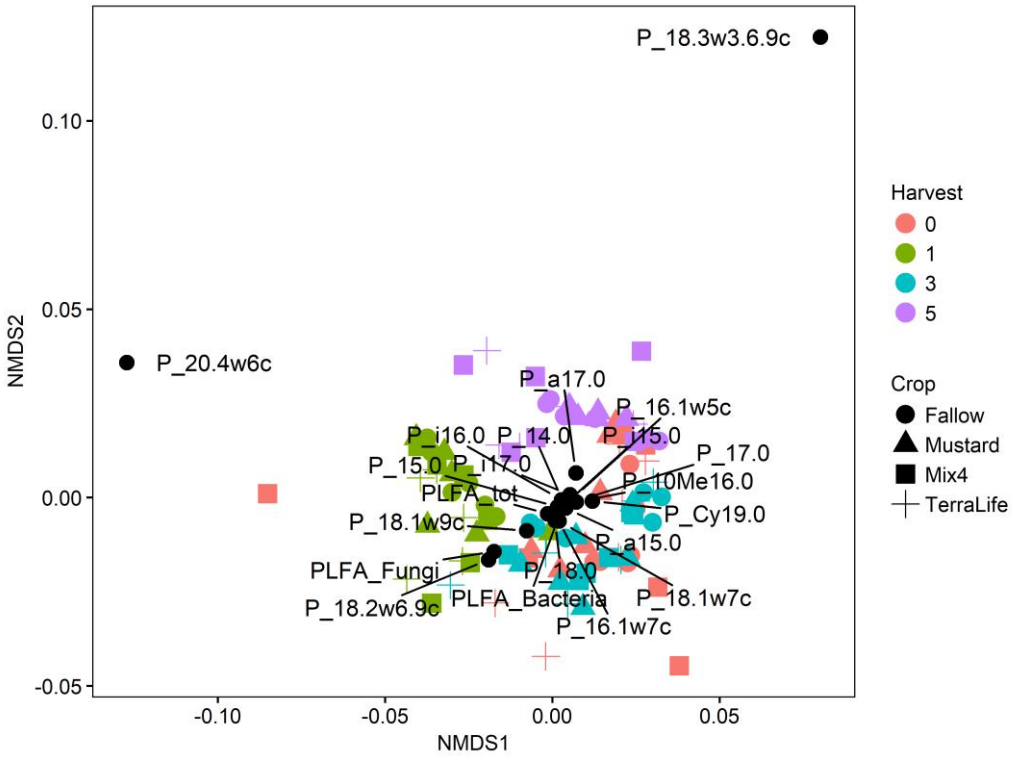

Figure 3.12. Relative PLFA amounts for all soil samples (regardless of depth) after applying NMDS ordination method.

3.4. Excess ^{13}C in catch crop soil PLFAs

In figures 3.13., 3.14. and 3.15. differences among the different catch crop treatments are presented combining PLFA and excess ^{13}C content in these. This technique has been referred to as stable isotope probing (SIP) coupled with ^{13}C-PLFA (Moore-Kucera and Dick 2008, Willers *et al.* 2015).

In figure 3.13., excess ^{13}C in total PLFAs is shown. In the TerraLife´s shallow soil (0-10 cm) the content is significantly higher than in the Mustard´s as a whole, although the former presents a very high variability on the first day after labelling (which can be observed with the standard error bars). In the deeper soil (20-30 cm) the amount of excess ^{13}C in PLFAs is much lower and there are no significant differences among the catch crop treatments.

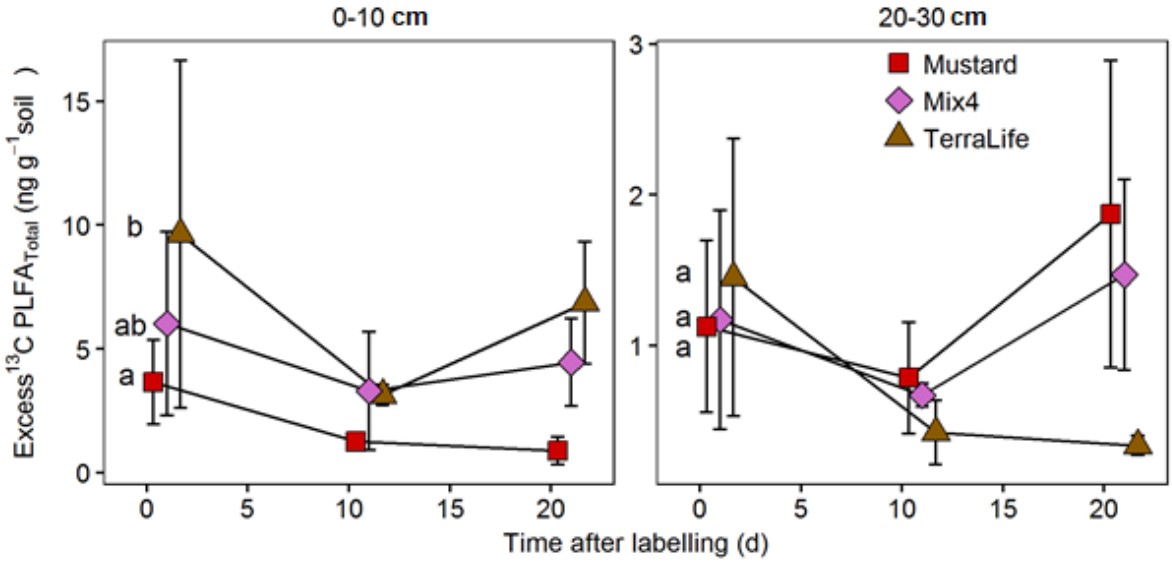

Figure 3.13. Excess ^{13}C of total PLFAs per catch crop treatment and sampling date from surface to 10 cm depth (left) and 20 - 30 cm depth (right). Note, values on the x-axes are presented as factors in order to avoid overlapping of symbols.

In figure 3.14. excess ^{13}C of the fungal PLFAs is presented. The TerraLife´s 0-10 cm soil has a significantly higher excess ^{13}C in the PLFAs extracted than that of the Mustard. Although values are an order of magnitude lower, in the deeper soil (20-30 cm), the Mix4 soil is the one that presents a significantly higher value than that of the Mustard.

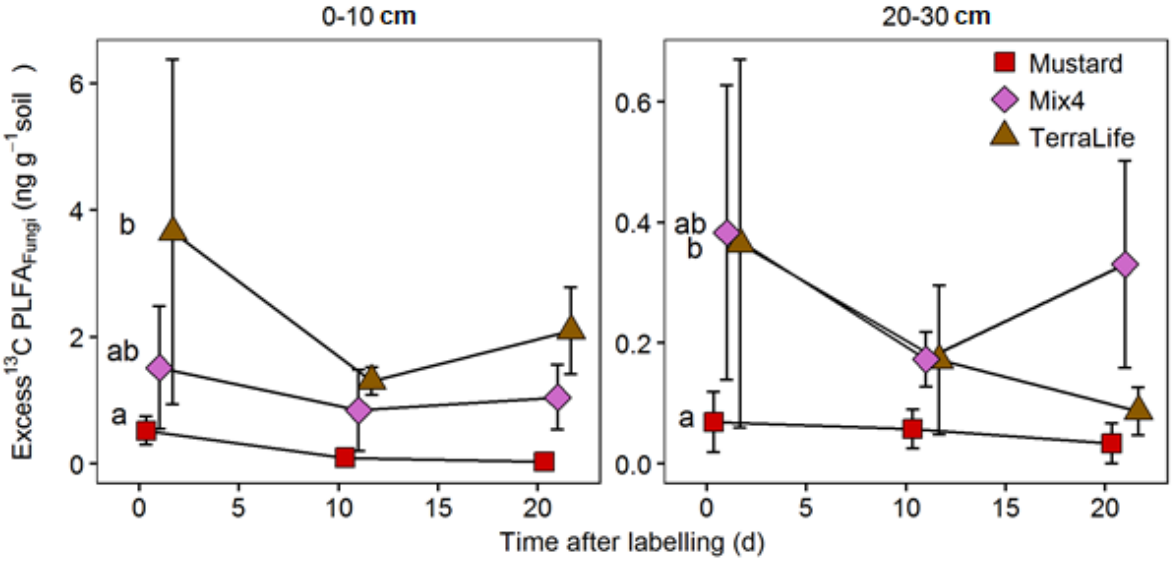

Figure 3.14. Excess ^{13}C of fungal PLFAs per catch crop treatment and sampling date from surface to 10 cm depth (left) and 20 - 30 cm depth (right). Note, values on the x-axes are presented as factors in order to avoid overlapping of symbols.

In figure 3.15., excess ^{13}C of the bacterial PLFAs is presented. There are no significant differences among catch crop treatments at both depths, although there is a noticeable increase in the Mustard´s value on the last sampling day at 20 – 30 cm depth.

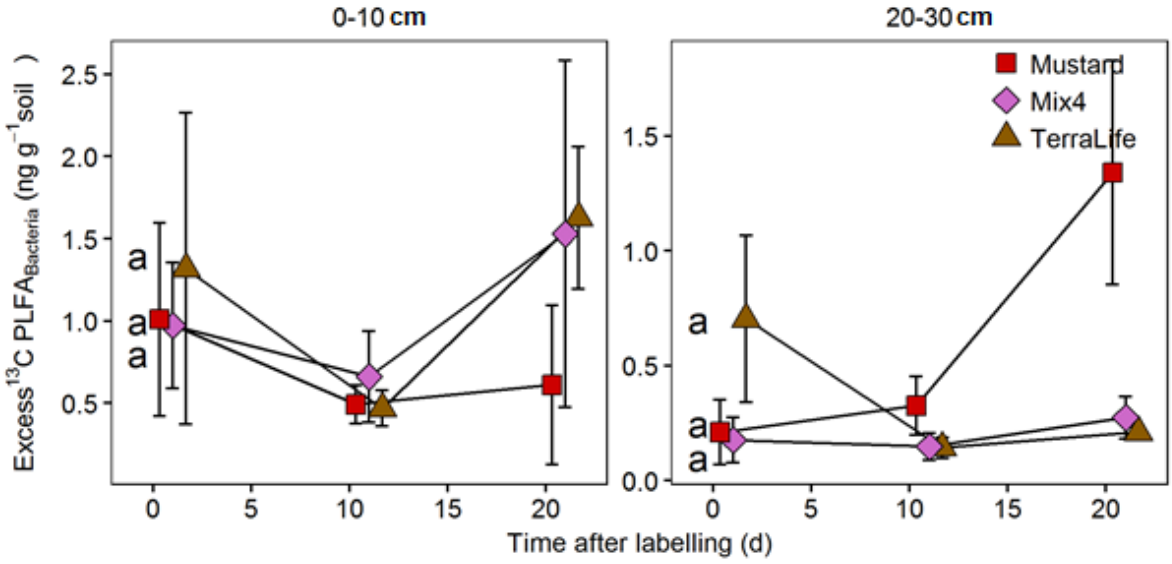

Figure 3.15. Excess ^{13}C of bacterial PLFAs per catch crop treatment and sampling date from surface to 10 cm depth (left) and 20 - 30 cm depth (right). Note, values on the x-axes are presented as factors in order to avoid overlapping of symbols.

4. Discussion

This study investigated the potential of the use of catch crops, comparing a single-species with two multi-species catch crops. The study uses techniques to observe atmospheric C fixation and soil microbiome composition and abundance.

After pulse labelling with $^{13}CO_2$, the presence of ^{13}C in the soil air at different depths was observed and it´s MRT at each depth calculated. The incorporation and presence of the isotope in living plant structures and the NEP of the different catch crops were also studied.

Total amount of PLFA of the different catch crops´ soils was compared after extraction. Also those PLFAs that were specific to AMF, Gram-negative bacteria, common to all fungi and common to all bacteria were analysed, and the Fungi/Bacteria ratio for the different soils was also calculated.

4.1. Atmospheric C fixation

The presence of the isotope ^{13}C in soil air was noticeably high on the day after labelling both in shallow (0 – 10 cm) and deeper soil (20 – 30 cm) and the values on the following sampling day (5 days later) present a clear drop, showing that the first ^{13}C pulse quickly disseminates or disappears from the soil air. The process in which the ^{13}C enters the plant-microbe-soil system and back to the atmosphere happens in a period of hours to days for all the catch crop soils. The results are consistent with those from similar labelling studies where, after observing a high concentration on the first day after labelling, the presence of the label decreases to a rather constant level within the following two weeks (Yao et al. 2015).

There were differences in the MRT at different depths being over 2 days for all soils at 10 cm depth and 17 days for the Mix4 and TerraLife soils at 60 cm depth. The significantly longer ^{13}C residence in the air of the deepest layer of the multi-species catch crop soils sampled may be due to a higher recycling of plant exudates by the soil microbiota, which has been observed that drives microbial composition (Morgan *et al.* 2005, Raaijmakers *et al.* 2009, Pulleman *et al.* 2012). Most likely, this effect was due to the different plant species rooting depths, that allows them to explore various different niches in the soil and support a more abundant and diverse microbiome. Pointing in the same direction, the MRT of the ^{13}C in the TerraLife soil regarding it´s flux from the soil surface, was significantly higher than in the other catch crops´ soils. This suggests that the catch crop with the highest plant diversity could be inducing a higher capacity for its soil to maintain newly incorporated C from the atmosphere. In other words, a higher C cycling and sequestration potential.

The significantly higher excess ^{13}C in the TerraLife´s plant shoots (when compared to those of Mustard) and roots (when compared to both Mix4 and Mustard) suggests that there was a higher incorporation of atmospheric C into its plant structures, which is consistent with the ^{13}C allocation data mentioned previously. The TerraLife mixture had a higher above ground phytomass, thus a higher leaf area, so the photosynthetic activity of the TerraLife when compared to the other catch crops was higher. This aspect, together with the release of exudates and litter decay (and further release back to the atmosphere due to microbial respiration) may also explain the higher newly incorporated C content in the soil.

To analyse the atmospheric C fixation due to plant activity in the different catch crops, NEP was calculated. From our results it can be clearly observed how NEP values increase from the fallow plots to the Mustard, from the Mustard to the Mix4 and finally from the Mix4 to the TerraLife; being this last one significantly higher than the fallow and the Mustard. This points in the same direction again,

the increase in catch crop diversity, strongly increases the NEP as a result of the increase the amount of C that is fixed from atmospheric CO_2 due to photosynthesis.

4.2. PLFA analyses

In the shallow soil (0 – 10 cm), TerraLife had significantly more abundance of soil fungal PLFAs than the fallow and the Mustard. One major difference between the single species catch crop and the diverse catch crops, is the presence of mycorrhiza forming plant species. Mustard's roots do not form mycorrhizal associations and thus have a much lower fungal presence in the soil. Higher plant diversity may have also boosted fungal presence because they are often less specific and are more mobile than bacteria by forming networks, both saprophytes and mycorrhiza.

Based on the results, the specific presence of AMF could not be considered significantly different among treatments, although higher in the TerraLife´s soil at 0 – 10 cm depth. AMF are ubiquitous in terrestrial ecosystems (Zhu and Miller 2003) and can establish symbiotic relations with many different plant species, which justifies its similar presence in all the studied catch crop soils. This again may be due to the diversity of plants in the mixture. AMF have been considered important in soil C storage acting as a middle-actor for C uptake, even increasing C fixation of the plants with which it establishes symbiotic relations (Zhu and Miller 2003). The higher amount of atmospheric C taken up by the TerraLife catch crop cannot be explained by a higher presence of AMF in the soil in this case. However, if we consider the influence above ground communities may have on those bellow (Wardle et al. 2004), specific plant AMF partnerships could contribute to higher photosynthetic C incorporation.

However, when considering the Fungi/Bacteria ratio based on the PLFAs extracted, the Mix4 soil and the TerraLife´s are significantly higher than that of the Mustard and fallow plots in the shallow soil. This means that the multi-species catch crops have a relatively higher proportion of fungal PLFA biomarkers or a relatively lower proportion of bacterial PLFA biomarkers. Bacterial dominated soils are generally more disturbed, with high nutrient availability and less soil organic matter; whereas fungal dominated soils correspond to those which are less disturbed, have less nutrient availability and higher organic matter content in temperate natural soil systems (van der Heijden, 2008). Our soil belongs to a conventional agricultural system, with high nutrient input and high levels of disturbance, in which the soil food web is mainly driven by a bacterial-based energy channel, with tendency to loose nutrients from their cycle and accumulate little C in their soils (Wardle *et al.* 2004). The presence of higher fungal biomarkers in the TerraLife's shallow soil suggests that there is a system with a tendency to close the nutrient cycles within the soil and to increase C allocation (Zhu and Miller, 2003). We cannot say that there is a fungal-based energy channel, but there is however a higher presence of fungi within the soil implying a certain change or shift in the underground nutrient and C cycles, due to the effect of higher plant species diversity. In this way, higher plant diversity stimulates a higher fungal presence in agricultural soils, and it's potential positive effects on soil health and crop production.

Regardless of soil microbial diversity, based on our results, total soil PLFA was higher in the TerraLife soil, which was significantly higher than the fallow soil unlike the other two catch crops. Higher plant species diversity implies higher diversity of potential interactions with soil microbes (van der Heijden, 2008). The TerraLife catch crop, acting as a species diverse grassland, can establish many different interactions with soil bacteria, depending on the species, but it can also be favouring the

presence of fungi that form networks in the soil interacting symbiotically with various the plant species. Due to this diversity of plant species, together with the competition of different plant rhizospheres for nutrient acquisition and the higher NEP of the TerraLife, both bacterial and fungal abundance have been boosted whether it is near a specific plant's root or across the field plot.

Additionally, if we look at the PLFAs of the 0-10 cm depth layer there are two different patterns. On one hand, the fallow soil and the soil covered with mustard do not have a drastic increase or decrease throughout time, whereas on the other hand, the Mix4 and TerraLife soils showed an increase in their PLFAs towards the last sampling days, which coincides with a decrease of temperature (see figure 2.3.). It is generally accepted that microbial activity decreases with temperature. However, when temperature decreases, before reaching freeze stage, soil microorganisms respond to cold stress by a series of physiological changes (Schimel *et al.* 2007, Willers *et al.* 2015). One of these is converting saturated fatty acids from their membranes to unsaturated fatty acids to maintain their membranes fluidity, assuring their survival at lower temperatures (Pietikäinen *et al.* 2005, Chattopadhyay 2006 (2); Schimel *et al.* 2007). This could somewhat explain the increase in the PLFA profile, but it does not explain the differential behaviour of mustard and fallow soils when compared to the Mix4 and TerraLife. Although, if the fact that fungal growth happens to be less inhibited by lower temperatures than bacterial growth (Pietikäinen *et al.* 2005) is taken into consideration and that mustard does not form mycorrhizal associations, the PLFA peak towards the last sampling days, could be explained as a change in mycorrhizal fungi membrane PLFAs as a response to a drop in temperature.

4.3. Conclusions

Planting catch crops to reduce N loss in agricultural systems in wet temperate climates provides positive results but other aspects are difficult to assess (Thorup-Kristensen *et al.* 2003). The plant species selected for these have a very important impact on the soil's microbial community (Wardle *et al.* 2004) thus influencing biogeochemical cycles. Changes in the C flux and allocation as well as microbial abundance and diversity give us an idea of other beneficial effects diverse catch crops have on the soil.

The results of this study supports that NEP increases with catch crop diversity. In our case, more atmospheric C was fixed by the multi-species catch crops than by the single-specie catch crop (mustard, *Sinapis alba* L.), which was also contained in them. The improved C cycling, due to a higher C sequestration in diversified catch crops, stimulated microbial activity by the higher input of plant exudates to the soil microbiota.

There was a significantly higher abundance of total PLFAs and fungal PLFAs in the TerraLife soil at 0-10 cm depth when compared to the fallow soil. And, although not always significantly higher, there was a clear tendency for the more diverse catch crops to have a higher abundance of PLFAs. The results of the study suggest that microbial biomass increases with plant diversity

The relatively higher Fungi/Bacteria ratio in the multi-species catch crops when compared to the single-specie and fallow soil suggests that the multi-species crops manage to alter the microbiome in a different way than single-species, with a tendency to have higher fungal presence in the soils of plant diverse terrestrial systems. This is suggested to be due to the presence of mycorrhiza forming plant species and the higher degree of heterogeneity of rhizospheres in the soil. Thus, based on the results,

the soil microbiome composition is different when various species of plants growing together than when grown as a monocrop.

The use of the catch crops analysed with higher plant diversity in the field stimulates microbial abundance and diversity as well as increasing atmospheric C fixation. Further research must be carried out, especially to study specific species interactions and using other species. However, from our findings and the available literature on the subject, diverse catch crops seem to have a greater potential in agriculture than exclusively reducing the loss of nutrients, increasing atmospheric C fixation, boosting the soil microbiome and the services they provide plants and thus, the services they provide humans.

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